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THE BIOCHEMICAL JOURNAL

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I. A STUDY OF THE VARIATIONS IN THE CHEMICAL COMPOSITION OF NORMAL HUMAN COLOSTRUM AND EARLY MILK.

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A very extensive literature has grown up during the past half century, on the subject of the chemistry of normal human milk. So vast has this literature become, that the task of absorbing and correlating its conclusions into a coherent understanding of the subject as a whole has become one of extreme difficulty and laboriousness. On the other hand, some attempt to do so must form the basis of any new work. From a review of the main communications in the literature, a list of which is appended, certain facts stand out and can, we suggest, be made the basis of an attempt to explore the matter in greater detail.

1. We have reliable and accurate knowledge of the general nature of the main constituents of human milk, and adequate methods for their estimation.

2. We have on record a very large number of observations of milk at all stages of lactation, and are aware of the changes most usually found.

If, however, we review the aggregate of facts presented by points 1 and 2, an admirable consideration of which appeared in a communication last year [Gardner and Fox, 1925], the main feature of the picture is the exceedingly wide range of values shown in normal milk by each constituent. These variations complicate our understanding of the composition of normal milk, and put a serious bar to any study of the conditions of abnormal milk. Furthermore, the great bulk of the work done hitherto on normal milk has not only been done abroad, but has also been achieved through the study of wet nurses—that is, of women accustomed to a specialised routine for the production and extraction of milk. As their conditions differ radically from those of ordinary nursing mothers, it cannot safely be assumed that the lactation results will necessarily be the same.

The present paper is the report of an attempt on the basis of work already known to deal with both these positions, to investigate in ordinary normal mothers the factors determining the variations in the chemical composition of their milk.

Method and Material.

The period of lactation chosen for investigation was the first 14 days after parturition, the reason for this choice being the accessibility to continuous observation of women in a labour ward, and the scantiness of our knowledge of this period. The mothers selected were all normal women, in-patients of the Obstetric Unit of the Royal Free Hospital. Among these patients were some who had been admitted to the V.D. Section of the Obstetric Unit for gonorrhoea. No mother with a history of syphilis was included.

In the vast majority of previously published communications the milk for analysis was obtained from single samples of the milk of different women. As by this procedure no opportunity is given for the observation of individual differences or their bearing upon general results, our procedure has been instead to make continuous observations of a number of individual women throughout their stay in the maternity wards.

Every sample was collected personally in the wards by one of us (M. F. L.), and taken to the London School of Medicine for Women where the analyses were carried through on the same day by S. T. W., M. B. and E. I. T. In only two instances was the milk kept overnight.

Chemical methods.

The constituents examined were protein, sugar, ash, fat, calcium and solids. Our aim at each point has been, not so much to achieve absolute percentages, as to arrive at some idea of the grounds underlying the differences that we found. The following are the methods of chemical estimation employed.

Protein. The total nitrogen was estimated by the Kjeldahl-Gunning method. 2·5 cc. of milk were usually taken for analysis, and the protein precipitated by saturated tannic acid solution, but where the amounts of milk available for examination were small, 1·5 cc. or occasionally 1 cc. only was used for the determination of N. In all cases where possible, the estimations have been done in duplicate with appropriate blank experiments.

Sugar. The method of Folin and Denis [1918] was employed. Calculation of the sugar content from the figures obtained from titration is according to the following empirical formula given by Folin and Denis:

$$\frac{4{\cdot}04 \times \text{dilution}}{\text{titration}}.$$

Fat. The method of Gottlieb [1892] has been used, 5 cc. of milk being usually taken; but where the amount of milk available did not allow of this quantity, the maximum amount that could be given was diluted to 5 cc. with water before extraction of fat. In all these estimations the term "fat" is used to denote all the ether-extractable substances.

Solids and ash. 2·5 cc. milk were measured into a weighed platinum basin; the fluid was evaporated on a water-bath for half-an-hour and then transferred to a steam-oven and heated to constant weight. The solid residue was converted into ash by ignition at low temperature, the basin never being allowed

to show a red glow. In some cases a duplicate estimation was made by just charring the solid residue, dissolving out the salts with water and filtering through a small tared filter. The carbon and filter paper after drying were ignited together, the filtrate added, evaporated to dryness and gently ignited. No difference in result was obtained by these two methods.

Calcium. A modification of the McCrudden [1912] method for small quantities of calcium, as elaborated for the estimation of calcium in blood [Widdows, 1923] was adopted. All the precautions which were taken in the estimation of calcium in blood serum were also observed here. In addition the milk was diluted (1 in 10) before use for estimation, to render the percentage of calcium comparable with that of serum. The results were calculated to g. of calcium oxide per 100 cc.

Clinical methods.

Very early in the investigation it became quite clear that the method of extraction of the milk had a profound bearing upon the composition of the sample obtained. No uniform technique was therefore adhered to, but the method was varied in each instance according to the problem under consideration. A note of the technique employed, therefore, accompanies each group of figures, the symbol *P* being equivalent to the use of a breast pump, *H* signifying digital expression. For the main part, and unless otherwise stated, the samples were collected between 10 a.m. and 11 a.m. and in immediate relation to the 10 a.m. or 10.30 a.m. feed. Altogether 164 samples were investigated—wholly or partly—of 13 women. That is, 5 women through 8–13 days and 8 women through 2–6 days.

Results.

It has been the custom in most papers of this kind to print the values, found for all the constituents of the milks examined, in a single table. By this means, the exact composition of each sample of milk can be seen. However, it has been found that the behaviour of the different constituents in the results to be demonstrated could not be clearly shown unless they were considered separately. The figures in the column headed No. are the laboratory labels of the sample of milk from which the specimen was taken and afford a means of correlation between the various tables. Each of the constituents observed will therefore be examined in order, according to its behaviour during the period under observation and its reaction to circumstances local to the taking of the individual sample of milk.

Protein. In the consideration of the protein content of the milk, 4 women were studied for 11 days, and 8 women for 4–8 days, and Fig. 1 represents the values found. The curves for *primiparae* are shown in an unbroken, and those for *multiparae* in a broken line.

It will be seen that in every case the highest protein percentage is to be found in the first 3 days after parturition, and that the level falls steeply during the first week to reach an approximate average of 1·4 % by the end

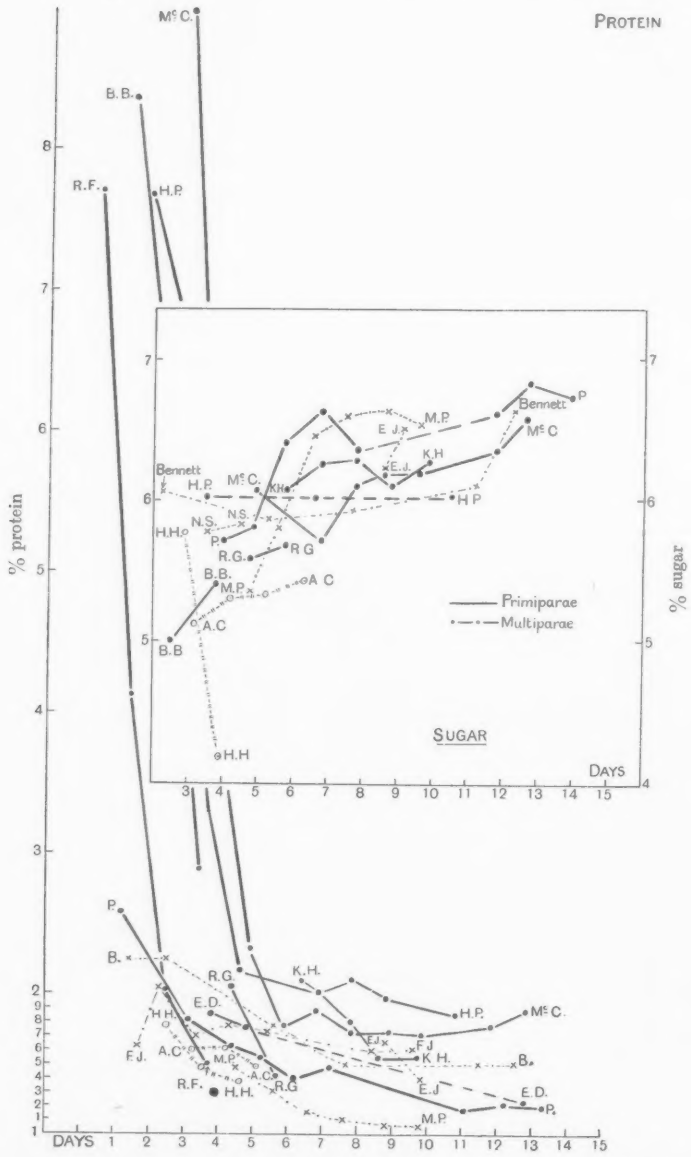
PROTEIN
% protein
% sugar
DAYS
SUGAR
Primiparae
Multiparae
Mc C.
B.B.
R.F.
H.P.
Bennett
M.P.
E.J.
K.H.
N.S.
H.H.
P.
R.G.
A.C.
E.D.
F.J.

Fig. 1.

of the 13th day. F. J. is the one exception. It may further be observed that as far as this small number of cases is concerned the initial protein values for *primiparae* appear to be much higher than those for *multiparae*. Moreover the curves followed by the *multiparae* show a quicker fall than those of the *primiparae* and reach a lower constant level. These observations are borne out by the protein values given by Adriance [1897] and Carter and Richmond [1898] if their tables are arranged in this way.

It is also possible that the quantity of milk available for the sample may be of significance in modifying the percentage of protein in it. The volumes obtained for McC., B. B. and H. P., by expression or when the amount taken by the baby (ascertained by test weighing) is considered in addition, do not exceed 12 cc., whereas with F. J. and M. P. the minimum amount obtained was 30 cc. and rose several times to 60 cc. or 90 cc. On the other hand, a percentage of 2·9 has been found on the 5th day in a sample of 105 cc. and 1·74 in a sample of 9·7 cc. It would appear therefore that the total volume of fluid in the breast may correlate with the percentage of protein found in the milk though this consideration, as is shown later, does not seem to influence the sugar or the calcium. It is worthy of note also that in most cases it is those individuals who show a high protein content in their early milk who have also a high value for total ash.

Sugar. If the sugar values for individual women are plotted in a graph in the same manner as those for protein, a similarly definite relationship between them becomes apparent. This, within certain limits, appears to be constant for all women. That is to say, that apart from any other consideration, the day of lactation upon which a sample of milk is taken will have a marked and definite relationship to the amount of sugar to be found in the milk. The graph (Fig. 1) represents the course taken by the sugar values of 11 cases through 2–11 days. It will be noticed that the lowest values are to be found in the early days of lactation and that these rise irregularly throughout the first 14 days. The direction of the curve taken is, however, opposite to that of protein. No such very low sugars were found by us as by some other observers, our lowest percentage exclusive of case H. H., whose other constituents also showed abnormalities, falling only to 4·5 %. Unfortunately, no sample for sugar estimation was obtained earlier than the 3rd day after parturition.

As regards comparison between the milk of *multiparae* and *primiparae*, although it would appear possible on the whole that that of *primiparae* shows a somewhat lower sugar value than that of *multiparae*, yet no constant rule is observable for this.

Ash. After as large a quantity of milk had been obtained at a single feed as the circumstances would permit, such estimations of the ash were carried out upon it as the volume would allow. For the purpose of satisfactory ash analysis the collection of the whole 24 hours' milk is strongly advised by Holt, Courtney and Fales [1915], but, as our interest was in the development of the

mineral content in individual women through a period of days, rather than in the exact constitution of the ash itself, this procedure could not be adopted. The quantity of milk upon which the analysis was done was in most cases 2·5 cc., though in a few cases a somewhat smaller volume had to be taken. Altogether 73 samples of 9 women were investigated representing 2 *multiparae* and 7 *primiparae*. The values obtained are represented in Fig. 2, plotted according to the average results for each day obtained for each individual for the period under observation.

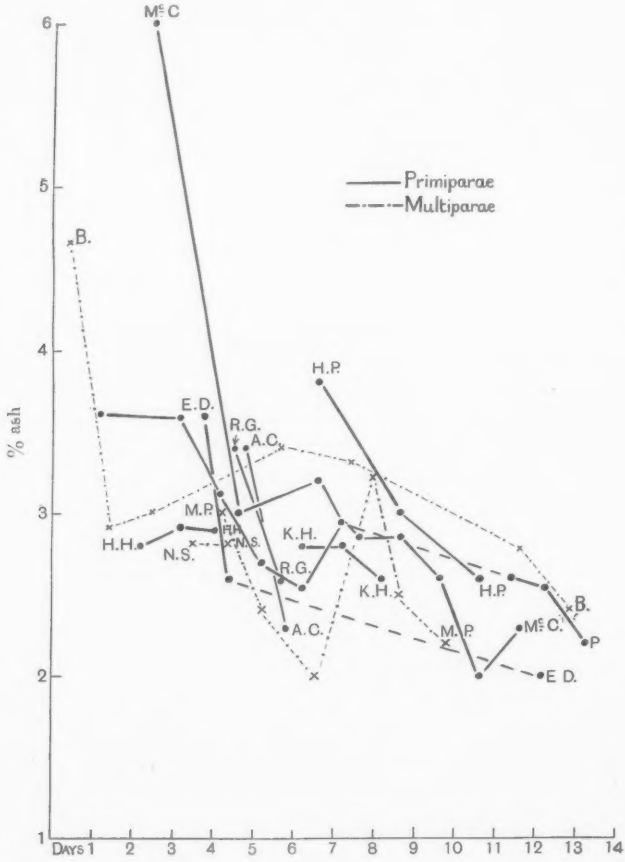

Fig. 2.

It will be seen that the course of the mineral constituents tends to show the same type of variation as that of protein and sugar, the direction of the curve, however, is analogous with that of the protein. Unfortunately the

number of samples of the milk of *multiparae* is very small and therefore no true comparison can be instituted between the curve of these and the *primiparae*. The tendency, however, seems to be in accordance with that of protein, that is, that the higher percentages appear in the early milk of *primiparae* rather than of *multiparae*. The fall is by no means so regular as that of the protein, and there are certain unexplained swings to be noticed in many curves. In the one example of continuous work on early ash found in the literature [Woodward, 1897], the same swinging values are found, although the figures are on the whole lower than our percentages. All the other analyses are unfortunately of single specimens of different women and so not susceptible of comparison.

The next point for general consideration is the question of the possible *variation in the composition of milk during the course of a single feed.* As a means of investigating this point the following two procedures were adopted. In the first, a sample of milk was taken (either by hand or with a breast pump) before the child was fed, the child was then put to the breast for a varying period of time and finally a sample was taken by hand or pump after it had sucked. By the alternative method the baby was allowed to take his whole feed from the one side, while an equal or larger amount was taken simultaneously or immediately after from the other and analysed in fractional portions, the "first" and "third" of these corresponding to the "before" and "after" of the other method. The composition of the milk of either sample was investigated with regard to the percentage content in it of each constituent in turn. Table I represents these values for protein (*i.e.* N × 6·37) and sugar in percentages.

Table I. *Variations in single feed. Protein and sugar.*

With baby							Without baby						
	Sample in cc.		Before		After			Sample in cc.		First		Last	
No.	B.	A.	Sugar	Protein	Sugar	Protein	No.	F.	L.	Sugar	Protein	Sugar	Protein
16/ 17	30	30	5·47 *P*	2·05 *P*	5·70 *P*	2·20 *P*	30/ 31	21	27	5·31 *P*	1·51 *P*	5·46 *P*	1·50 *P*
88/ 89	6·3	6·3	4·93	1·95	5·62	2·14	33/ 34	26	26	5·47 *P*	1·31 *P*	5·49 *H*	1·33 *H*
141/142	22	25	5·94	1·60 *P*	5·77	1·70 *P*	45/ 47	29	29	6·78 *Dr*	0·96 *Dr*	6·39 *H*	0·98 *H*
143/144	30	15	5·94 *P*	1·60 *P*	5·68 *P*	1·74 *P*	116/117	55	25	5·95 *P*	1·53 *P*	5·58 *P*	1·53 *P*
86/ 87	2·6	6·3	—	1·78	—	2·09	119/120	27	30	4·08 *P*	1·37 *P*	4·45 *P*	1·37 *P*
138/139	5·0	5·5	—	2·50 *H*	—	2·67 *H*	160/161	22	40	6·90 *P*	1·14 *P*	6·80 *H*	1·23 *H*
146/147	23	24	—	1·49	—	1·33	168/169	3	11	5·64 *H*	2·24 *H*	5·65 *H*	2·24 *H*
149/150	22	12	—	1·57	—	1·43	174/176	53	30	6·56 *H*	1·21 *H*	6·65 *H*	1·21 *H*
126/127	6	6	6·11 *H*	—	5·31 *H*	—	40/ 42	30	30	6·53 *H*	—	6·53 *H*	—
134/135	9·5	11	6·40 *H*	—	5·90 *H*	—	49/ 51	30	31	—	1·12 *Dr*	—	1·12 *H*
186/187	20	11	—	1·42	—	1·49	111/112	25	13	5·54 *P*	—	5·44 *P*	—
152/153	14	8	7·11 *P*	—	6·14 *P*	—	101/102	12	20	—	1·16 *Dr*	—	1·16 *P*

As regards the *protein* it will be seen that in the columns representing the values found before and after a feed there is a larger range of difference than in that of the milk obtained from a complete emptying of one breast, the highest being 0·31 % which represents approximately 14 % of the total value found. On the other hand, in the latter case practically all the samples are stationary since differences of 0·01 % and 0·02 % are, in this case, within

the limit of experimental error. If any constant variation therefore can be said to occur in the protein, it is in the direction of a rise in the "after" specimen.

With regard to the *sugar* it will be noticed that in the section "With baby," 5 show a decrease, 2 an increase, and none is stationary. In the other section, 4 show a decrease, 3 show an increase and 3 are stationary. Both the more frequent and, with one exception, the maximum variations it will be seen are in the downward direction, which is in line with the suggestion already made by Denis and Talbot in this matter [1919], that the sugar content of the milk of the first part of a feed is higher than that of the last. This is to be seen most clearly in the dripped samples (*Dr*). The method of extraction of the milk appears to have no influence upon the sugar values.

With regard to the *comparison of samples taken from either breast*, under as nearly as possible similar conditions, out of 12 samples examined for protein, 6 showed no difference, and the difference in the others (0·1 % to 0·2 %) when taken in association with the fact that the sample from the one breast was of the whole quantity, and the other from the sum of that taken before and after feeding, is probably relative to the variation already considered between the early and late milk of a single feed. With regard to the sugar content, in those instances in which both breasts, at the time at which the sample was taken, were entirely normal, no difference appeared.

As concerns the *ash* with regard to the variations in a single feed and the comparison of the milk of either side, such differences as were found to exist do not follow any rule. The number of our examples here, however, is too small to be of significance.

Fat. It has been seen that protein, sugar and ash show variations in percentage content according to the day of lactation. With fat this factor is not operative. If a list of fat values for a number of cases be written down in sequence, according to the date after parturition, no order is discoverable in their values. In the cases considered some milks showed 1 % fat on the 4th day and 7 % on the 7th, or 7 % on the 2nd and 2 % on the 11th. There were *primiparae* who showed a range of 0·6 % to 2 % and *multiparae* giving 2·9 % to 5·9 %. But if, on the other hand, instead of the day of lactation, the variations in a single feed come to be considered, a consistent picture develops.

In representing the amounts taken by the baby, in certain instances, as for example No. 84/85, only the total amount taken from both breasts was known, and as for the purpose of this table only one breast was relevant and the exact amount taken from that side is not discoverable, the quantity, therefore, taken by the baby is represented by the total amount from both sides divided by 2.

In Table II the following points will be noticed.

1. There is a rise in fat content in all samples but one, and this rise in all cases, other than those specially marked, is more than 1 % and can reach

as high as 5·8 %. If moreover, omitting the cases starred, the average be taken of the differences in the section "With baby" it will be found to be higher than that of the first and last samples.

2. The greatest difference in both sections appears in those cases (marked *Dr* and *H*) where the first sample was of milk that had dripped from one breast while the baby fed from the other, and the last sample was taken by hand.

3. In all cases but four (marked *) the method of procedure was either to take the first sample with a breast pump and to finish by digital expression, or to use the same technique for the collection of both samples. In examples 78/79, 88/89, 156/157 and 172/173 the usual procedure was reversed, the first part of the specimen being taken by hand and the last by pump. In the first three of these the result has been to reduce the difference normal for these individuals (1·1 %, 5·4 % and 1·3 % respectively) to 0·2 %, 0·6 % and 0·4 %, whilst in case 172/173 it has fallen to − 0·6.

Table II. *Variations in single feed. Fat* (%).

With baby									Without baby							
	Sample in cc.					Ozs. taken by				Sample in cc.						
No.	B.	A.	Preg.	Day	Before	baby	After	Difference	No.	F.	L.	Preg.	Day	First	Last	Difference
186/187	20	11	*P*	4	1·52 *HP*	2·25	2·86 *HP*	+1·3	116/117	55	25	*P*	4	1·62 *P*	2·83 *P*	+1·2
141/142	22	25	*P*	5	3·04 *P*	1·50	4·56 *P*	+1·5	119/120	27	30	*P*	5	0·64 *P*	2·42 *P*	+1·8
143/144	30	15	*P*	5	1·92 *P*		4·16 *P*	+2·2	33/ 34	26	26	*M*	6	3·55 *P*	5·98 *H*	+2·4
146/147	23	24	*P*	6	1·89 *P*	0·75	2·93 *P*	+1·1	7/ 8	21	15	*P*	6	3·10 *P*	5·28 *P*	+2·1
149/150	22	12	*P*	6	2·76 *P*	1·25	6·15 *P*	+3·4	*156/157	26	15	*P*	8	3·90 *H*	4·37 *P*	+0·4
*78/ 79	1·5	5	*P*	7	5·44 *H*	1·50	5·60 *P*	+0·2	45/ 47	29	29	*M*	10	2·24 *Dr*	5·51 *H*	+3·3
						2										
82/ 83	1·8	2·5	*P*	8	4·36	2·25	6·30	+2·0	49/ 51	30	31	*M*	10	2·92 *Dr*	6·12 *H*	+3·2
84/ 85	2·8	3·3	*P*	8	3·64		9·02	+5·4	160/161	22	40	*P*	13	4·32 *P*	5·58 *H*	+1·2
*88/ 89	6·3	6·3	*P*	9	4·64 *H*	0·75	5·20	+0·6	160/161	22	40	*P*	13	4·32 *P*	5·58 *H*	+1·2
						2										
102/103	12	11	*P*	13	0·60 *Dr*	0·5	6·45 *H*	+5·8	*172/173	22	5	*M*	4	4·96 *H*	4·36 *P*	−0·6

All these milks were taken after the 3rd day of lactation. When the fat for these 3 days was examined it was found that in many cases its behaviour was reversed, the fat content being higher in the first sample than in the last. This is shown in Table III. But if the percentages for these milks are carefully scrutinised it appears that this reversal of values occurs only in the case of *primiparae.*

Table III. *Early milk. Fat* (%).

	Sample in cc.					Ozs. taken by			Sample in cc.					Ozs. taken by	
No.	B.	A.	Preg.	Day	Before	baby	After	No.	B.	A.	Preg.	Day	Before	baby	After
178/179	1·7	1·5	*P*	1	0·95 *H*	Nil	0·53 *H*	71/ 72	1	2	*P*	2	7·14	0·25	5·02
138/139	5	5·5	*P*	2	3·13 *H*	0·5	2·72 *H*	73/ 74	0·7	1·6	*P*	2	6·92		5·74
181/182	1·7	2·3	*P*	2	1·66 *H*	0·25	1·26 *H*	80/ 81	1·5	5	*P*	7	9·98	1·5	8·66
														2	
166/167	4·5	11	*M*	2	3·50 *H*	1·25	6·05 *H*	16/ 17	30	30	*M*	3	1·57 *P*	—	2·12 *P*
						2									
170/171	3·5	38	*M*	3	2·97 *H*	—	4·08 *H*	168/169	3	11	*M*	3	3·75	—	4·39

Now since it is known that the fat in milk is present in the form of an emulsion and is therefore liable to respond, as to its degree of emulsification, to changes in physical conditions, it would appear reasonable that these conditions should play a large part in the determination of the resultant fat percentage in the milk. A consideration of the above figures suggests that the percentage of fat in milk depends upon two factors, the one inversely and the other directly. The first of these is the quantity of liquid available as a suspensory fluid. In a very full breast just before a feed, when stimulation of the one side produces dripping from the other, the fluid will be at a maximum, and therefore, according to this point of view, the fat should be at a minimum. This is exactly what in fact occurs—see cases 102/103, 45/47 and 49/51 (Table II). On the other hand, at the end of a feed when the breast is near exhaustion, the fluid will be at a minimum, and one would expect, therefore, a rise in fat content, and, as will be seen by the above tables, this is in every instance the case. The second factor in operation is the question of direct force applied to the breast itself. This is absent in the case of a milk that drips, at its least in the gentle use of a breast pump, and at its maximum in vigorous digital expression or the action of the jaws of a strongly sucking baby. The influence of this factor is direct upon the proportion of fat in the resulting milk, that is to say, in an opposite direction to the action of the fluid factor. The percentage of fat resulting in the milk depends, therefore, upon a balance of these two forces, the fat rising at the end of a feed if all other conditions are equal. But if greater pressure be applied at the beginning than at the end this rise can be very much modified (78/79, 88/89, 156/157) or even altogether obliterated (172/173).

As a further experiment in confirmation of these contentions, in one case a first sample was taken by vigorous finger expression and set aside. A second sample was then taken by very gentle use of the pump, and finally the remaining milk was withdrawn by hand. The values of the fat found were, first sample 3·88 % (*H*), second 4·08 % (*P*), last 5·34 % (*H*). That is to say, that although the sample with 5·34 % of fat followed immediately upon the sample with 4·08 % and was not very much in excess in quantity, yet the fat difference was 1·26 %. To illustrate both factors at one time, in two other samples a "before" and "after" specimen was taken by pump and hand on the 4th day from the left breast 6 hours after the last feed on that side, and the estimated fat content was 1·62 % and 2·86 % respectively, and the total amount secreted $3\frac{1}{4}$ ozs. ($2\frac{1}{4}$ ozs. sucked and 1 oz. expressed). The whole available quantity of the right breast, where the baby had fed 3 hours before, expressed by a pump, reached a total of $1\frac{3}{4}$ ozs. with a fat content of 2·9 %. That is to say, that on the one side the factor of digital expression plus that of the end of the feed operating in a large quantity of fluid resulted in a fat percentage just equal to that appearing in a very much smaller quantity obtained by the gentler method of expression from the other side.

As the procedure hitherto adopted for the study of the fat content of milk

by most observers has been either to mix the fore milk and strippings [Denis and Talbot, 1919], or to take the middle milk [Adriance, 1897; Woodward, 1897], it was thought to be of interest to investigate in a series of cases the fat content of these relative to each other. Table IV represents the figures obtained, the second column being the fat percentage in a sample composed of equal parts of first and last milk and the third that of the middle milk of the same sample.

Table IV.

No.	A.+C.	B.	No.	A.+C.	B.
23/23 *a*	2·36	1·88↘	24/24 *a*	3·70	3·69→
26/26 *a*	5·15	5·12→	27/27 *a*	4·56	4·78↗
28/28 *a*	4·23	4·51↗	29/29 *a*	3·98	3·63↘
38/39	3·34	2·25↘	37/37 *a*	3·65	3·47↘
44/44 *a*	4·19	4·46↗	43/43 *a*	4·26	3·65↘

The arrows at the side of the columns indicate by their direction the relative values of the fats of these two columns. It will be seen that the relationship between them is fortuitous.

It is postulated, therefore, that, whereas the other constituents of the milk so far considered vary in percentage according to the general influences of the development of lactation, the percentage of fat depends alone upon factors local to the breast and the manner of extraction of the sample.

Calcium. The calcium content of the milk of 18 women was estimated over a period of 2–15 days and according to the same schema as with the other constituents. The following table shows the results obtained in 5 typical cases ranged according to days after parturition.

Table V. *Values for calcium in the milk of 4 women.*

Name	No.	Preg.	Volume of sample cc.	Day	CaO %
McC.	121	*P*	14	6	0·0342
	122/127	„	5	7	0·0409
	128/129	„	16	8	0·0375
	130/132	„	10	9	0·0457
	133/134	„	15	10	0·0448
L. P.	140	*P*	57	4	0·0543
	146/151	„	20	6	0·0502
	152/155	„	16	7	0·0502
	158	„	40	12	0·0491
	160/161	„	30	13	0·0463
H. P.	72/ 74	*P*	2	2	0·0465
	76	„	10	4	0·0498
	77/ 81	„	6	7	0·0466
	82/ 85	„	2·5	8	0·0492
	86/ 89	„	5	9	0·0501
	90/ 91	„	20	11	0·0465
R. J.	15	*M*	30	2	0·0427
	16/ 17	„	30	3	0·0430
	19/ 22	„	30	4	0·0424
	23/ 28	„	30	5	0·0491
	29	„	30	10	0·0510

NOTE. The figures for calcium given above represent the average figure for all samples estimated on the given day.

It will be seen that no variation is perceptible according to the day of lactation as far as the period 2–15 days is concerned. This naturally does not in any way prejudice the possibility of a later rise and fall with the later development of lactation, as has been suggested by Schabad [1911] and Hunaeus [1909]. It will be seen also that there is a definite regularity in content of the milk of the individual women themselves. This has been the case with all but one of the women investigated, the maximum range of variation among all the samples taken of any one woman being from 0·009 % to 0·017 %. In one case, however, a greater variation occurred, the lowest figure reaching 0·0399 % and the highest 0·628 %, this over a range of 5 days and 20 samples. This can be accounted for either by the hypothesis of Schabad [1911], that such a degree of variation does occur in a certain proportion of women, or by the possible inaccuracies introduced into the work by the use of a breast pump. It would therefore appear that the difference in calcium values shown between the milks of individual women during the period under observation is far greater than that occurring among the samples of milk of any single individual. This fact was also found by Schabad for the milk of the later periods of lactation.

As regards the question of the influence upon the calcium content of the place of the sample in a feed, it has been suggested by Schabad [1911] and Hunaeus [1909] that the value of the calcium falls at the end of a feed. In order to study this point, 22 samples were investigated of the first and last milk of a feed and of that taken before and after a feed. Three specimens were also analysed fractionally from breasts that had dripped. The results are given below:

Table VI. *Variations in single feed. CaO* (%).

With baby				Without baby			
No.	Before	After	Difference	No.	First	Last	Difference
84/ 85	0·0481	0·0516	+0·003	49/ 51	0·0458 *Dr*	0·0595 *H*	+0·014
86/ 87	0·0600	0·0487	−0·011	111/112	0·0369 *P*	0·0333 *P*	−0·003
88/ 89	0·0566	0·0571	+0·001	116/117	0·0369 *P*	0·0369	—
131/132	0·0456 *H*	0·0453 *H*	—	119/120	0·0380 *P*	0·0333 *P*	−0·005
146/147	0·0476	0·0476	—	160/161	0·0447 *P*	0·0442 *P*	—
149/150	0·0521	0·0513	−0·001	40/ 42	0·044 *H*	0·044 *H*	—
152/153	0·0477 *P*	0·0441 *P*	−0·003	30/ 31	0·0570	0·0626 *P*	+0·005
16/ 17	0·0410 *P*	0·0450 *P*	+0·004	45/ 47	0·0410 *Dr*	0·0485 *H*	+0·007
101/103	0·0343 *Dr*	0·0433 *H*	+0·009	33/ 34	0·0588 *P*	0·0485 *H*	−0·010

If the range of experimental error be taken to be approximately 0·003 % it will be seen that 11 samples show no variation while 5 show a rise and 2 a fall. If, on the other hand, the figures be taken as they stand, 5 are stationary, 7 show a rise and 6 a fall. It would appear therefore, that such differences as occur, are either fortuitous in direction or negligible in amount. We would suggest that, since Schabad gives only 3 samples, and Hunaeus 6, a selection of such as, for instance, 119/120, 86/87 and 33/34 would account for their findings.

When the calcium content of the milk of either breast was compared, the variations found were as follows: in 8 out of 17 cases there was a variation of 0·003 % or under, in 6 out of 17 cases a variation of 0·004 % to 0·008 %, in 3 out of 17 cases variations respectively of 0·014 %, 0·013 % and 0·023 %; for this latter instance no reason is apparent.

In comparing the values for calcium with those for ash, it is worthy of note that the high ash values found at the beginning of lactation are not represented in the calcium. The excess of mineral in the colostral and early milk must therefore be due to some component other than calcium, possibly magnesium, which, according to Holt, Courtney and Fales [1915], is variable throughout lactation and highest in the colostrum period (considered as 1–12 days) as also are potassium and sodium.

Solids. The total solids were estimated in 67 samples from 16 women, and show a variation of from 7·6 % to 18·9 %. But if one case showing 7·6 % and a single example of 18·9 % be omitted, the range of the remaining samples is 9·76 % to 14·98 %. The height of the figures for solids is found consistently throughout all samples to vary in general relationship to the fat and to give an average mean value of 12·68 %.

It is to be noted that the estimated total solids have not, in a considerable number of cases, reached the same total as an arithmetic addition of the results for sugar, fat, protein and ash would give for the same milks, the difference between the two varying from plus 0·046 % to plus 2 %, with a mean of 1·18 %. This is in agreement with the work of Camerer and Söldner [1896] and earlier observers, who found in the case of colostrum and early milk a similar discrepancy. In working with later milk Söldner did not find this discrepancy to occur, and suggested, therefore, that its appearance in the early milk could not be due entirely to experimental error, as had hitherto been suggested, but must represent actual unanalysed substances in the milk. In the analyses recorded in this paper only the precipitable protein nitrogen was estimated. The soluble bodies containing nitrogen therefore, which are known to exist in milk, would necessarily form part of the discrepancy found. Furthermore, the extractives containing no nitrogen which are known to be present in milk, and citric acid, have not been considered.

To sum up, it is suggested that the usual view of milk, as a secretion homogeneous in its response to stimuli, cannot be maintained, and indeed lies at the root of a number of the contradictions and obscurities found in the results of milk analyses. On the contrary, in agreement with the view first put forward by Forster [1881], it is contended that milk is composed of elements opposite in behaviour and very varied in their response to identical stimuli.

Conclusions.

Our conclusions with regard to the chemical composition of milk of the first 14 days of lactation and the factors influencing its variation may be summarised as follows.

1. There appears to be a difference between the milk of *primiparae* and *multiparae* with regard to the behaviour, in the early days of lactation, of the protein and fat.

2. The protein and ash content of the milk vary regularly according to the day of lactation, being very high in *primiparae*, less so in *multiparae* on the 2nd and 3rd days, and falling steeply during the first week to reach an approximately normal level of from 0·98 % to 1·3 % for the protein, and 0·20 % to 0·25 % for the ash.

3. The sugar content varies also according to the day of lactation, but inversely as the protein, and less regularly, being at its lowest in the early days and rising irregularly to a normal level of approximately 6·5 % by the end of the second week.

4. The fat content of the milk varies only according to the physical conditions of the breast and the actual extraction, and not in relation to the day after parturition, but in *primiparae* the general behaviour is reversed in the first days of lactation.

5. The calcium content of the milk varies up to the 15th day between individual women, but not according to any factors concerned with individual lactation and is uninfluenced by factors local to the breast.

6. There is some possibility that the protein content of the milk rises slightly at the end of a single feed, the difference being greater when the samples are taken in relation to an actual feed than when only the first and last portions of an artificially emptied gland are considered.

7. The lactose content of the milk appears to be higher at the beginning than at the end of a feed.

8. The total solids vary roughly as the fat, and show a range from 9·6 % to 14·98 %.

We wish to acknowledge our gratitude to Mr Alfred Langton whose generosity, in conjunction with that of the Medical Research Council, has made the research possible. To Prof. Louise McIlroy for putting the material at our disposal and for encouragement in the carrying out of the work, and to Prof. Winifred Cullis for very helpful advice and criticism, we wish to offer our warmest thanks. It is also a pleasure to put on record our appreciation of the able and generous cooperation of Miss Hill, M.B., Third Assistant of the Obstetric Unit, and of the Nursing Staff of the Royal Free Hospital, in the carrying out of the work.

REFERENCES.

Adriance (1897). *Arch. Pediat.* **14**, 22, 85.
Camerer and Söldner (1896). *Z. Biol.* **33**, 42, 535.
Carter and Richmond (1898). *Brit. Med. J.* i, 199.
Denis and Talbot (1919). *J. Dis. Child.* **18**, 93.
Folin and Denis (1918). *J. Biol. Chem.* **33**, 521.
Forster (1881). *Ber. deutsch. chem. Ges.* **14**, 591.
Gardner and Fox (1925). *Practitioner*, **114**, 2, 153, 680.
Gottlieb (1892). *Landw. Versuch. Stat.* **40**, 1.
Holt, Courtney and Fales (1915). *J. Dis. Child.* **10**, 229.
Hunaeus (1909). *Biochem. Z.* **22**, 442.
McCrudden (1912). *J. Biol. Chem.* **10**, 187.
Schabad (1911). *Jahrb. Kinderh.* **74**, 514.
Widdows (1923). *Biochem. J.* **17**, 34.
Woodward (1897). *J. Exp. Med.* **2**, 217.

II. A NOTE ON THE SIGNIFICANCE OF GELATIN FOR BACTERIAL GROWTH.

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In a study of the effect of various substances present in meat extracts on growth and peroxide formation in pneumococcal cultures it was found that no growth of the strains used occurred in peptone water, and only occasionally in bouillon. The addition of small amounts of Coignet's "Gold Label" gelatin to either of these media always resulted in abundant growth. Previously, Robertson, Sia and Woo [1924] had found that the period of survival of pneumococcus can be considerably increased if 0·1 % gelatin be added to suspensions of these organisms in water or saline. Gelatin purified by Loeb's method also had a beneficial effect on the pneumococcus. The results of the writer's work suggest that 0·1 % commercial gelatin would have some nutritive value for the organism. This is also the view of Meleney and Zau [1924] deduced from the effect of small amounts of gelatin on haemolytic streptococci in saline suspensions.

The growth of pneumococcus in Parke Davis peptone water containing various percentages of gelatin was determined. 3 % gelatin proved to be most suitable. Higher concentrations, up to 16 % gelatin, caused an increase in the duration of the lag period of growth, but viable organisms were present after 3 weeks' incubation. In peptone water containing less than 3 % gelatin, decreases in the amount and in the period of growth were observed.

A 20 % solution of "Gold Label" gelatin, adjusted to p_H 7·8 was autoclaved for periods varying from 20 minutes to 20 hours. No difference in the amount of growth of pneumococcus could be observed in peptone water containing 5 % gelatin from these autoclaved solutions. In the absence of peptone and on the addition of 0·5 % sodium chloride, growth was only obtained in a 15 % gelatin solution autoclaved for 20 hours, and, to a lesser extent, in the sample autoclaved for 18 hours. Growth was also possible in concentrations of 5–10 % of the sample autoclaved for the longest period.

A sample of the commercial gelatin was purified by washing at p_H 4·7 according to the method of Loeb [1922], hydrochloric acid being used instead of acetic acid. All the washings were concentrated *in vacuo*, most of the acid being removed in this process. It was found that growth of the pneumococcus occurred in peptone water containing either the concentrated washings or

the washed gelatin. A mixture of these fractions, in approximately the same proportion as in the original sample of gelatin, had an effect on growth similar to that of the untreated gelatin. Growth was also possible in bouillon on the addition of 2 % purified gelatin.

A portion of the concentrated washings was dialysed in a parchment shell against distilled water at 2°. The concentrated dialysate was found to promote growth of pneumococcus in 2 % washed gelatin-peptone media whilst the dialysed solution had no effect. The ash of "Gold Label" gelatin and of the washings used above have an influence on the growth of the organism similar to that of the dialysate. Furthermore, it was repeatedly found that the addition of 4 % of a Ringer-Locke solution to peptone water allows growth of the pneumococcus to take place in peptone water when washed gelatin is present.

It may be stated in view of the above observations and of other evidence obtained in the course of this investigation that relatively complex protein decomposition products are required for the growth of the pneumococcus. These may be supplied by bacteriological peptones (preferably with a low free amino-acid content) or by hydrolysed gelatin. Secondly, the addition of purified gelatin will allow or will increase growth of pneumococcus in peptone water or in bouillon. Thirdly, in "Gold Label" gelatin there are important impurities which are dialysable and not destroyed by incineration. Ringer-Locke solution may be substituted for these substances.

The following medium was prepared in order to determine whether the recognition of the three foregoing conditions is sufficient to satisfy the growth requirements of the pneumococcus. A 10 % solution of purified gelatin was partially hydrolysed by autoclaving for 24 hours at 120°. Growth of pneumococcus was obtained in a portion of this medium containing the ash of the washings. The media were inoculated with a small amount (0·1 to 2·5 cc. medium) of a dilute suspension of the organism in saline.

Preliminary experiments have been carried out to test the effect of gelatin on the growth of some of the more delicate bacteria other than pneumococcus. Two strains of *B. diphtheriae* were found to grow in a 3 % gelatin-peptone medium and not in either bouillon or peptone water. Better growth of a strain of meningococcus and of two strains of streptococcus was obtained in the gelatin-peptone than in bouillon or peptone water, but the strains of gonococcus examined would not grow in any of these media.

It should be pointed out that the direct assimilation of gelatin by pneumococcus is improbable as this organism does not liquefy gelatin, nor has it, according to Avery and Cullen [1920], any enzyme capable of hydrolysing proteins. Of the many physical properties of gelatin solutions which may affect the growth of bacteria, the decreased solubility and rate of diffusion of oxygen are of particular interest in the case of pneumococcus. Gillespie [1913] has recorded the fact that an inoculum must contain more pneumococci to produce growth in a liquid than on a solid medium. It may be that, before

the initiation of growth of the pneumococcus, a certain minimum value of oxygen tension is essential. This condition, however, is probably realised by the reducing action of the organisms if the area inoculated be sufficiently limited by physical conditions such as obtain in gelatin solutions.

To Prof. J. W. M'Leod, in whose laboratory this work was carried out, I wish to express my thanks for advice and for facilities.

REFERENCES.

Avery and Cullen (1920). *J. Exp. Med.* **32**, 547.
Gillespie (1913). *J. Exp. Med.* **18**, 584.
Loeb (1922). Proteins and the Theory of Colloidal Behaviour, p. 35. (New York.)
Meleney and Zau (1924). *J. Exp. Med.* **39**, 811.
Robertson, Sia and Woo (1924). *J. Exp. Med.* **39**, 199.

III. PEROXIDE FORMATION BY PNEUMOCOCCUS AND ITS RELATION TO BACTERIAL OXIDATION-REDUCTION REACTIONS.

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Avery and Morgan [1924] in confirming and extending the findings of M'Leod and Gordon [1922] have summarised the nature of the conditions which must be maintained in order that maximum amounts of peroxide may be produced in cultures of pneumococcus. No mention is made, however, of the effect of the various constituents of the culture media on the amount of peroxide formed, though experiments reported in a later paper [Avery and Neill, 1924, 2] show that peroxide is not formed by washed pneumococci, nor by extracts of washed organisms, unless there is present a substance of undefined character which may be obtained from cell washings, meat or yeast. The organism is usually grown in media which contain substances of animal or vegetable origin, so that, with an adequate supply of oxygen and in the absence of peroxide-destroying agencies, considerable amounts of peroxide have generally been obtained. Pneumococcus does not grow in peptone water, but it has been shown [Platt, 1927] that the addition of a small amount of "Gold Label" gelatin to peptone water provides a medium in which the organism will yield a growth of the same order as that obtained in the presence of meat extract, as, for example, in 3 % gelatin-bouillon. The amount of peroxide produced when pneumococcus is grown aerobically in the former medium is considerably smaller than when the same medium containing meat extract is used. This difference is apparently due to the absence from gelatin-peptone of the substances necessary to complete the peroxide-forming mechanism of the organism. A convenient medium is therefore available for the purpose of determining the nature of these components.

With reference to the function of the "complementary" substances, Avery and Neill [1924, 2] suggest that "the principal rôle may be the furnishing of easily oxidised or autoxidisable substances." In a later publication [Avery and Neill, 1924, 3] they show that the complementary substances are thermostable, and, like the thermolabile component in extracts of washed pneumococci, do not react with molecular oxygen or methylene blue. This opinion has been modified [Neill and Avery, 1925], for it is stated that the thermostable substances react slowly with molecular oxygen forming oxidising agents, and, in anaerobic conditions, methaemoglobin and methylene blue are reduced to

a slight extent. The extract of washed pneumococci is said to catalyse a reaction which is represented by the expression:

$$\underbrace{C}_{\text{Thermolabile cellular substance}} + \underbrace{RH}_{\text{Thermostable easily oxidised substances}} + O_2 \rightarrow \underbrace{ROOH}_{\text{Oxidising agent}}$$

Neill and Hastings [1925] and Neill [1925, 2] find that, whilst both oxygen consumption and haemoglobin oxidation by the autoxidisable substances of alcoholic extracts of meat infusion and potato juice are greatly accelerated by the cellular catalyst (C), the reaction with other autoxidisable substances, *e.g.* turpentine, linseed oil and oleic acid, is not affected by the presence of (C). In view of the results of the present research it appears that the substances (RH) required to complete the oxidation-reduction system (C + RH) of the pneumococcus are not mainly the autoxidisable substances of extracts of animal and vegetable tissues of the type found in turpentine. Indeed, were they similar, one would expect that the reactions of both systems would be accelerated alike on the addition of component (C). The suggestion therefore is made that the extracts used, supposed to contain the substances (RH), also contain compounds capable of acting as hydrogen donators. In the presence of the pneumococcus substance (C), hydrogen may be donated to molecular oxygen, which may need to be activated, with the subsequent formation of hydrogen peroxide. Given appropriate conditions, haemoglobin may be oxidised to methaemoglobin and, in the absence of oxygen, methylene blue and methaemoglobin may be reduced. An important hydrogen donator in the case of peroxide-forming bacteria appears to be lactic acid, which may be present in media containing meat or yeast extracts or may be produced by the organism from fermentable substances in the medium.

Experimental.

Methods.

The estimation[1] of peroxide in the cultures was carried out by adding $M/100$ titanous sulphate in excess, the amount of reagent not oxidised being determined by titration with $M/100$ iron alum solution in the presence of potassium thiocyanate. The conditions specified by Thornton and Chapman [1921] for accurate estimations have been observed. It was found to be more convenient to store the titanous sulphate solution in $4N$ sulphuric acid under hydrogen than to adopt the method advanced by Russell [1926]. All the titrations were carried out in an atmosphere of carbon dioxide. The accuracy of the method for the present purposes was proved by estimating known amounts of peroxide in such culture media as would be employed. Small initial titrations, which remained unchanged on prolonged incubation, were observed in

[1] I am indebted to Mr C. R. Hoskins, B.Sc., of Leeds University, for his assistance with the technique of the peroxide estimations.

sterile media containing meat extract. The titration values for sterile control media are subtracted from those obtained in the following experiments.

The number of viable organisms in a culture was determined by dilution of various amounts with 0·1 % gelatin-saline, several loopfuls of each dilution being plated out on a heated blood-agar medium.

For the preparation of the media Parke-Davis' peptone and Coignet's "Gold Label" gelatin were used. The bouillon was prepared by extracting lean beef with water at 100° for 2 hours (1 l. water to 500 g. beef) and adding 1 % Parke-Davis' peptone and 0·5 % sodium chloride. The media were all adjusted to p_H 7·8 and sterilised by autoclaving. The strain of pneumococcus (pn. 4) was isolated from pus from a case of empyema and was found to be bile-soluble.

Exp. 1. 240 cc. of 3 % gelatin-peptone and of 3 % gelatin-bouillon were placed in 3-litre Erlenmeyer flasks, the depth of the medium being about 12 mm. 10 cc. of a 10 hours' culture of a strain of pneumococcus (pn. 4) were used to inoculate each medium. Samples were taken out at intervals, under sterile conditions, for estimations of growth and peroxide. The results obtained are expressed graphically in Fig. 1, where the logarithm of the number of viable organisms per cc. of culture and the amount of peroxide (expressed as cc. $M/100$ H_2O_2) per 10 cc. of culture are plotted against the time of incubation at 37°.

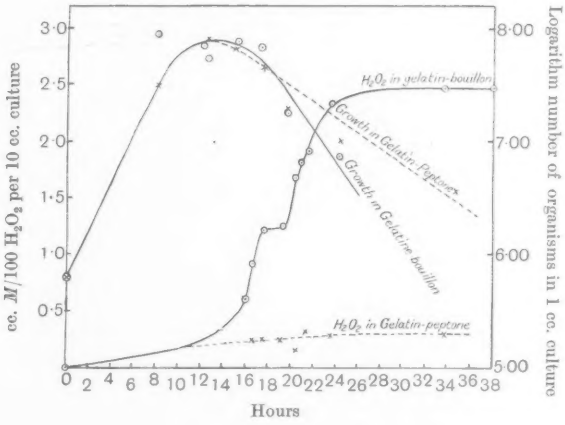
cc. M/100 H2O2 per 10 cc. culture
Logarithm number of organisms in 1 cc. culture
H2O2 in gelatin-bouillon
Growth in Gelatin-Peptone
Growth in Gelatine bouillon
H2O2 in Gelatin-peptone
Hours

Fig. 1.

It is evident from Fig. 1 that there is a marked difference in the amount of peroxide formed in the two media used, without corresponding differences in the amount of growth. Moreover, peroxide is not produced in gelatin-bouillon in appreciable amounts until near the end of the period of logarithmic increase of the bacteria. Another feature is more clearly demonstrated in the

following experiment when the cultures were incubated at a temperature lower than the optimum for growth.

Exp. 2. 10 cc. of 3 % gelatin-bouillon, inoculated with pn. 4 from an 18 hours' culture on heated blood-agar, were pipetted into 250 cc. Erlenmeyer flasks. After incubation at 30° for varying periods, 0·5 cc. was used for a growth estimation, the remainder being titrated with $M/100$ titanous sulphate and $M/100$ iron alum solutions. The results are given in Fig. 2.

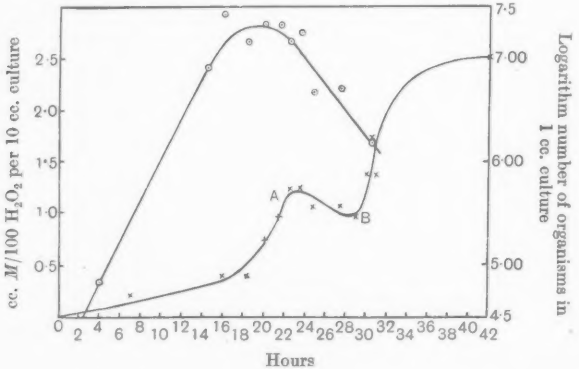

Fig. 2.

The portion (*A*–*B*) of the time-peroxide curve given in Fig. 2 appears to be related to the period of stationary growth of the organism and may be due to the effect of the gelatin on the regulation of the oxygen supply to the organism. The organisms which develop before and at the stationary period will exhaust the supply of oxygen, so that there is insufficient for the formation of more peroxide, and there is also a tendency to reduction of that already formed. The latter tendency ceases with the active growth of the organism, whilst the respiration of the autolysed or autolysing bacteria proceeds until the maximum value is attained. That peroxide may be formed in cultures under conditions incompatible with active growth has been demonstrated by Avery and Neill [1924, 1]. Also, inhibitory amounts of peroxide are found in gelatin-bouillon cultures before the maximum value (about 0·01 %) is attained. (Morgan and Avery [1924] show that growth of pneumococcus may be inhibited for 24 hours in a concentration of peroxide of 0·006 %.) There is evidence that more peroxide is formed by the organism than that represented by the actual titration values.

Exp. 3. 150 cc. each of 5 % serum-bouillon and 5 % serum-3 % gelatin-bouillon, inoculated from a serum-bouillon culture of pn. 4, were incubated at 37° in 1-litre Erlenmeyer flasks. The ox-serum, which had been sterilised by filtration, was heated to 60° for 30 minutes before use. The data obtained for the rate of growth and peroxide formation are represented in Fig. 3.

From these results it appears that the chemical and physical properties of serum are such as to yield more immediate and regular production of peroxide. The addition of gelatin to serum-bouillon however results in a time-peroxide curve of the type obtained for gelatin-bouillon. It will be seen that the "step" in this curve appears earlier on account of the larger number of organisms produced, as shown in the growth curve.

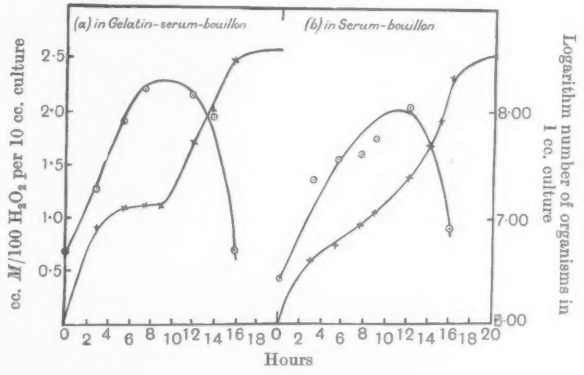
(a) in Gelatin-serum-bouillon
(b) in Serum-bouillon
cc. M/100 H_2O_2 per 10 cc. culture
Logarithm number of organisms in 1 cc. culture
Hours

Fig. 3.

Exp. 4. In Table I are given the results of some of a number of estimations of peroxide carried out on 10 cc. of 48 hour cultures of pneumococcus in 3 % gelatin-peptone containing sodium lactate. There was no marked difference in the amounts of growth in the various cultures after 12 hours' incubation at 37°.

Table I.

Media			$M/100$ H_2O_2
3 % gelatin-peptone			0·35 cc.
,,	+0·02 %	sodium lactate	0·78
,,	+0·05 %	,,	1·10
,,	+0·1 %	,,	1·22
,,	+1·0 %	,,	1·10

It is noteworthy that no peroxide is obtained in gelatin-peptone cultures of pneumococcus when a small amount (0·2 %) of sodium thiolactate is present, even when positive tests for peroxide are obtained in control cultures. This substance did not appear to affect the amount of growth.

In a 48 hours' culture of the pneumococcus, containing initially 1 % glucose, a titration of 0·95 cc. $M/100$ H_2O_2 was obtained. It will be observed that the peroxide formed on the addition of sodium lactate or glucose to gelatin-peptone cultures is not more than 50 % of the amount usually found in gelatin-bouillon cultures. Small increases in peroxide are found if 1 % glycogen or 0·02 % inositol is present in gelatin-peptone cultures. The meat extract no doubt furnishes other substances from which, directly or in the

course of metabolism, peroxide is produced. Quastel [1926] finds that the effect of mixtures of hydrogen donators is equal to the sum of the activities of the separate constituents. The probability is that a similar relationship exists for the constituents of a medium taking part in peroxide formation.

Discussion.

In addition to the foregoing observations, there are other lines of evidence which point to the important part played by lactic acid in bacterial oxidation-reduction reactions [cf. M'Leod, Gordon and Pyrah, 1923]. A comparison of the curves in the above figures, which are typical of a number obtained, with that given by Morgan [1924] for the rate of change in p_H of a culture of pneumococcus also suggests that the peroxide and acid production are related. Further, it has been observed that aerobic cultures, in which there is active peroxide formation, invariably develop a more acid reaction than those in which only small amounts of peroxide are produced. This difference is not determined by the amount of growth. Valentine [1926] has recorded interesting variations in the properties of two strains of streptococci X and Y. Strain X produces much less peroxide and less acid than strain Y. The latter strain has also a much wider range of fermentation reactions.

There is undoubtedly a close parallelism between the reducing and peroxide-forming activities of extracts of pneumococcus [Avery and Neill, 1924, 3], so that it may reasonably be assumed that under appropriate conditions lactic acid will complete the reducing as well as the peroxide-forming systems. A comparable mechanism appears to exist in washed *B. coli* which, in the presence of lactic acid, has a marked reducing action [Quastel and Whetham, 1925, 1]. No peroxide formation however will be evident, on account of the presence of catalase in *B. coli*. It is significant that yeast reacts towards lactic acid in a similar manner and that both yeast and *B. coli* appear to metabolise lactic acid only when an ample supply of oxygen is available.

Glucose has been found to promote peroxide formation in gelatin-peptone cultures of pneumococcus. Now Neill [1925, 1] has shown that glucose does not activate extracts of washed pneumococci, but only completes the oxidation-reduction system of the intact cell. It therefore appears to be necessary that, before glucose can function as a hydrogen donator, fission of the glucose molecule must occur, probably with the formation of lactic acid. Quastel and Whetham [1925, 2] find that glucose has a high "reducing coefficient" in the presence of resting *B. coli*. Whether in this instance the glucose undergoes metabolism cannot be determined from available data.

In conclusion, it is suggested that, taking into account the relation of lactic acid to the type of bacterial reaction under discussion, less lactic acid would be formed in aerobic cultures of lactic acid-forming bacteria than in anaerobic cultures. This may explain the recent results of Rona and Nicolai [1926] and of Neuberg and Gorr [1926].

SUMMARY.

1. Cultures of pneumococcus have been obtained, which, though aerated and free from catalase, yet produce only small amounts of peroxide. A considerable amount of peroxide is formed when cultures contain meat extract. This difference in behaviour is not determined by the amount of growth in the two media.

2. The rates of growth of, and peroxide formation by, pneumococci are compared under various conditions. The effect of oxygen supply on the rate of peroxide formation is discussed.

3. An increase in the amount of peroxide formed is observed in pneumococcal cultures containing lactic acid or glucose. It is suggested that the lactic acid functions as a hydrogen donator in the presence of the pneumococcus, peroxide being formed when oxygen acts as hydrogen acceptor.

4. The influence of glucose and of lactic acid on some reactions of pneumococci is discussed and correlated with observations on other organisms.

REFERENCES.

Avery and Morgan (1924). *J. Exp. Med.* **39**, 275.
Avery and Neill (1924, 1). *J. Exp. Med.* **39**, 347.
—— —— (1924, 2). *J. Exp. Med.* **39**, 357.
—— —— (1924, 3). *J. Exp. Med.* **39**, 543.
M'Leod and Gordon (1922). *Biochem. J.* **16**, 499.
M'Leod, Gordon and Pyrah (1923). *J. Path. Bact.* **26**, 127.
Morgan (1924). *J. Exp. Med.* **39**, 565.
Morgan and Avery (1924). *J. Exp. Med.* **39**, 335.
Neill (1925, 1). *J. Exp. Med.* **41**, 299.
—— (1925, 2). *J. Exp. Med.* **41**, 551.
Neill and Avery (1925). *J. Exp. Med.* **41**, 285.
Neill and Hastings (1925). *J. Biol. Chem.* **63**, 479.
Neuberg and Gorr (1926). *Biochem. Z.* **173**, 476.
Platt (1927). *Biochem. J.* **21**, 16.
Quastel (1926). *Biochem. J.* **20**, 166.
Quastel and Whetham (1925, 1). *Biochem. J.* **19**, 520.
—— —— (1925, 2). *Biochem. J.* **19**, 645.
Rona and Nicolai (1926). *Biochem. Z.* **172**, 82.
Russell (1926). *J. Chem. Soc.* **129**, 497.
Thornton and Chapman (1921). *J. Amer. Chem. Soc.* **43**, 91.
Valentine (1926). *J. Infect. Dis.* **39**, 29.

IV. A NOTE ON PLANT OXIDATION: THE NATURE AND REACTIONS OF THE SUBSTANCE "TYRIN."

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In an investigation of the oxidising systems of the potato, Szent-Györgyi [1925] obtained a preparation which, he states, plays an important rôle in this plant oxidation. This preparation, to which he gave the name "tyrin," is capable of "oxidation" to a red compound ("oxytyrin") by *ortho*-quinone or by the system potato oxidase-catechol. Szent-Györgyi represents the changes occurring in the potato as follows:

Mol. O_2 + oxidase + catechol → *o*-diketoquinone.
o Quinone + leuco-tyrin (reduced form) → catechol + oxytyrin (red compound).
Oxytyrin + "active" hydrogen → leuco-tyrin.

According to Szent-Györgyi, "tyrin" is concerned in the oxidations of the potato and possibly other plant and animal tissues, as similar preparations could be obtained from various mammalian and other tissues, and he concludes that "tyrin" can be classified as a member of Palladin's group of respiratory pigments.

Happold and Raper [1925] have shown that tyrosinase will act upon phenolic compounds in the presence of amino-acids to give deeply pigmented substances only when the phenol is likely to furnish an *ortho*-quinone on oxidation. In these cases, pigment formation is accompanied by deamination. *o*-Benzoquinone alone produced the same results as the system tyrosinase-catechol (phenol or *p*-cresol). On these grounds a satisfactory explanation was afforded of the liberation of ammonia and pigment formation in the Chodat *p*-cresol-tyrosinase and similar reactions.

In the light of the experiments of Happold and Raper it appeared possible that the properties and reactions of "tyrin" were partly or wholly due to amino-acids, peptides or compounds of these with carbohydrates [cf. Borsook and Wasteneys, 1925]. Preparations of "tyrin" made by Szent-Györgyi's method have all contained amino-nitrogen in appreciable quantity and it has been found that the "oxidation" reactions of these preparations can be accounted for by the presence of nitrogenous compounds. The reactions of "tyrin"—the formation of a red compound on treatment with *o*-quinone or the catechol-oxidase system, the reduction of this compound with NaHS

etc.—can all be produced to approximately the same extent with solutions of various amino-acids or peptone containing the same amount of nitrogen. Thus "tyrin" appears to be a mixture of nitrogenous compounds, especially amino-acids, and to have no other significance in oxidation processes.

Preparation and properties of "tyrin."

The method of preparation was that described by Szent-Györgyi [1925], the first product being a thick oily substance, from which, however, it has been found possible to prepare a white stable powder by precipitation from a methyl alcohol solution with ethyl alcohol. The powder obtained had all the properties of the original sticky mass. Szent-Györgyi records a negative Millon's test for tyrosine, but a small amount of this amino-acid has been detected in all preparations, when care has been taken to avoid excess of the reagent. The presence of a trace of tyrosine has been confirmed by the Folin and Denis colour reaction [1912].

Analysis.	Total nitrogen (micro-Kjeldahl)	5·7 %	3·6 %
	Free amino-nitrogen (formaldehyde titration)	1·4 %	1·3 %

Relationship between "tyrin" and amino-acids.

If the pigmentation of "tyrin" under the influence of *o*-quinone or the catechol-oxidase system is due to the amino-acids present, liberation of ammonia should occur as in the experiments of Happold and Raper [1925]. A definite increase in the ammonia content of the solution during this pigmentation was observed, the determinations being carried out wherever possible by two methods, the aeration method (after addition of potassium carbonate), and the permutite filtration method of Whitehorn [1923]. Experimental details have been described previously [Happold and Raper, 1925; Raper and Wormall, 1925].

Solutions of glycine, alanine and glutaminic acid containing the same amount of nitrogen as a solution of "tyrin" were prepared and the properties of all solutions compared in the following respects.

(*a*) Red pigment formation with *o*-quinone—this pigmentation was approximately equal in the case of "tyrin" and glycine and somewhat less in the other amino-acids.

(*b*) Red pigment formation in the presence of catechol and potato oxidase at p_H 6·4—similar results to those in (*a*).

(*c*) Pigmentation with *o*-quinone after treatment with neutral formaldehyde—"tyrin" and amino-acid solutions were treated with varying amounts of neutral formaldehyde, and the solutions neutralised and tested with *o*-quinone after the addition of buffer solution at p_H 6·4. All solutions still retained the power of reacting with the quinone to form red compounds.

(*d*) Pigmentation with *o*-quinone after treatment with nitrous acid—"tyrin" solutions and amino-acid solutions behaved identically, the ability to react with *o*-quinone being destroyed almost completely in every case.

From these results the conclusion has been drawn that amino-acids, free and combined, present in the "tyrin" are solely responsible for the red pigment formation with *o*-quinone or with the catechol-oxidase system. Equivalent amounts of some of the more reactive amino-acids or peptone will give pigment formation to the same extent. "Tyrin" appears to contain, among other substances, a mixture of amino-acids, some simple peptides and compounds of these with reducing sugars.

THE CATECHOL-OXIDASE SYSTEM. *o*-QUINONE AND PEROXIDE FORMATION.

Szent-Györgyi [1925] produced evidence that the guaiacum reaction obtained with potato oxidase in the presence of catechol is due to the formation from the catechol of *o*-quinone and that the enzymes present take no further part in the blueing of guaiacum. These conclusions are based on the fact that if potato oxidase is allowed to act on catechol for 10 minutes at 37° and the enzymes precipitated with methyl alcohol, the enzyme-free filtrate will blue guaiacum. *o*-Quinone prepared by the method of Willstätter and Müller [1908] gave the same blueing of guaiacum, was inactivated by excess of catechol and showed the same general lability as the substance formed in the catechol-oxidase reaction. Proof of the identity of the reactive substance formed in the enzyme reaction was not furnished, but, recently, evidence of the formation of *o*-quinones from phenolic compounds under the action of the tyrosinase oxidase has been published [Happold and Raper, 1925; Raper, 1926]. Szent-Györgyi therefore divides the blueing of guaiacum by the catechol-oxidase system into two phases:

(*a*) catechol + oxidase + oxygen → *o*-quinone,

(*b*) oxidation of guaiacum by the oxide of catechol (*o*-quinone),

for which no enzyme is required.

The removal of enzymes from the solution was effected by Szent-Györgyi by the addition of methyl alcohol and filtration; but this does not preclude the possibility of small amounts of peroxidase or oxidase being present in the filtrate. These experiments, with additional controls, have therefore been repeated, using a method which is easy to carry out and which does not appear to be open to this objection. Potato oxidase was allowed to act on a catechol solution at p_H 6·4 for 10 minutes, the solution extracted with ether and the ethereal solution tested with guaiacum and benzidine. Positive results were obtained, while all the controls—catechol alone, enzyme alone and catechol + heated enzyme—gave negative results. Since these experiments were carried out, Onslow and Robinson [1926] have also confirmed the observations of Szent-Györgyi, a method involving precipitation of the enzymes with "dialysed iron" being used. As these authors admit, however, the use of "dialysed iron" in connection with guaiacum is open to objection, although their control tests gave negative results.

Experimental procedure. The enzyme used in these experiments was prepared by the method used by Szent-Györgyi [1925] which is based on that of Onslow [1920].

The following solutions *A*, *B*, *C* and *D* were prepared:

	0·1 % catechol cc.	Phosphate buffer (p_H 6·4) cc.	Distilled water cc.	Potato oxidase mg.
A	2	5	3	100
B	—	5	5	100
C	2	5	3	—
D	2	5	3	100 (heated)

The solutions were incubated at 37° for 5 minutes, each solution was shaken thoroughly with 15 cc. pure ether, and the ethereal extracts were pipetted off and filtered. These ether extracts were tested in the following manner with an alcoholic solution of guaiaconic acid and 1 % alcoholic benzidine.

	A	*B*	*C*	*D*
3 cc. ether extract + 3 drops alcoholic benzidine	Blue	—	—	—
3 cc. ether extract + 3 drops alcoholic guaiaconic acid + 2 cc. distilled water	,,	—	—	—

These experiments have been repeated a large number of times and in no case did the controls give any colour. Specially purified ether has been used for the extractions, but this does not appear to be essential, for negative controls are obtained with once distilled ether. The blueing of guaiacum and benzidine in ethereal solutions appears to preclude absolutely the possibility of enzyme interference in this phase of the reaction.

In the course of an investigation of the production of peroxides from catechol and other phenols, solutions containing catechol and potato oxidase have been examined for hydrogen peroxide, with positive results. Onslow and Robinson [1926] have also demonstrated the production of hydrogen peroxide in such solutions and in solutions of catechol which had been allowed to oxidise by exposure to the air for several days.

This formation of hydrogen peroxide in the catechol-oxidase reaction, although slight, for most of that formed will quickly be decomposed by the catalase present or utilised by the peroxidase for oxidation purposes, can readily be demonstrated by the following method, which does not necessarily involve the use of ether. This solvent usually contains a peroxide which will give the titanium sulphate test for hydrogen peroxide and therefore has to be specially purified if used in connection with these tests.

Experimental procedure.

	1·0 % catechol drops	Phosphate buffer (p_H 6·4) cc.	Distilled water drops	Potato oxidase mg.
A	5	5	—	50
B	5	5	—	—
C	5	5	—	50 (heated)
D	—	5	5	50

These solutions were dialysed in parchment sacs against 20 cc. distilled water in each case. Samples (2 cc.) of each dialysate were withdrawn after 5, 10 and 30 minutes and tested for hydrogen peroxide by the addition of 0·5 cc. of 25 % H_2SO_4 and varying amounts of titanium sulphate in 5 % H_2SO_4. The presence of peroxide, presumably H_2O_2, in the dialysate from solution *A* only was indicated by a slight yellow coloration. A stronger reaction with the titanium sulphate reagent was obtained by carrying out the reactions on a larger scale, acidifying the dialysate with H_2SO_4 and concentrating under reduced pressure. The concentrated dialysates gave a yellow coloration only in the case of solution *A*. The slight yellow colour obtained with some of these controls was apparently due to the catechol, since no colour was produced if the solutions had been extracted previously with purified ether, as suggested by Onslow and Robinson [1926]. This treatment, however, in no way affected the positive peroxide tests with solution *A*.

Conclusions.

1. The "tyrin" preparation of Szent-Györgyi has been shown to contain appreciable amounts of amino-acids.

2. All the oxidative properties attributed to this preparation can be explained by the presence in it of free or combined amino-acids and can be imitated exactly by a mixture of amino-acids. "Tyrin" therefore plays no significant rôle as a respiratory pigment.

3. The suggestion made by Szent-Györgyi as to the mechanism of the blueing of guaiacum by the catechol-oxidase system has been confirmed by a method which precludes the possibility of enzymic action in the second part of the reaction. The production of hydrogen peroxide in addition to this oxidation product (presumably *o*-quinone), which blues guaiacum directly, has also been demonstrated.

REFERENCES.

Borsook and Wasteneys (1925). *Biochem. J.* **19**, 1128.
Folin and Denis (1912). *J. Biol. Chem.* **12**, 245.
Happold and Raper (1925). *Biochem. J.* **19**, 92.
Onslow (1920). *Biochem. J.* **14**, 535.
Onslow and Robinson (1926). *Biochem. J.* **20**, 1138.
Raper (1926). *Biochem. J.* **20**, 735.
Raper and Wormall (1925). *Biochem. J.* **19**, 84.
Szent-Györgyi (1925). *Biochem. Z.* **162**, 399.
Whitehorn (1923). *J. Biol. Chem.* **56**, 751.
Willstätter and Müller (1908). *Ber. deutsch. chem. Ges.* **41**, 2580.

V. THE MICRO-ESTIMATION OF CHLORINE IN WHOLE BLOOD SERUM OR CORPUSCLES.

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(*Received November 15th, 1926.*)

Previous methods.

Claudius [1924] described a method of micro-estimation of blood chloride in which he treated 0·2 cc. of blood with 20 cc. of $N/25$ silver nitrate and a few drops of nitric acid. After boiling this down to 0·25 cc. he completed the oxidation with potassium permanganate. Alcohol was added and the solution titrated with alcoholic ammonium thiocyanate. The use of alcohol was a considerable advance, but is open to the objection that it forms an explosive mixture with high concentrations of nitric acid. When titration is carried out in the presence of silver chloride, a large excess of nitric acid is necessary owing to the reversibility of the reaction

$$3AgCNS + FeCl_3 \rightleftharpoons 3AgCl + Fe(CNS)_3.$$

In addition, boiling down to small bulk is tedious and frequently causes loss by bumping.

Rehberg [1925] recently overcame these difficulties by maintaining a large excess of nitric acid, but keeping the reacting materials in small bulk. He utilised the special method of titration described by himself. The blood was digested with 100 volume hydrogen peroxide as introduced by Fowweather [1926] for blood-iron estimations. A single estimation of whole blood is completed in 2 hours, for the most part occupied by digestion in a water-bath.

The following method, using ammonium persulphate and nitric acid on 0·2 cc. of blood, gives a smaller margin of error for whole blood than Rehberg's method; it can be completed in 15 minutes for a single sample, or 12 minutes per sample over a series of estimations. It has the advantage that acetone intensifies the end-point more than alcohol and can be used in the presence of high concentrations of nitric acid.

Principle of the method.

The principle of this method is the destruction of the organic matter of the blood by ammonium persulphate and concentrated nitric acid, with the usual precipitation of the chlorine as silver chloride by excess of silver nitrate. The excess is titrated against alcoholic ammonium thiocyanate solution with concentrated ferric iron alum as indicator.

The titration is performed in the presence of acetone which intensifies the end-point by decreasing the dissociation of the ferric thiocyanate, and for a similar reason renders it more permanent. This enables a series of titrations to be brought to the same depth of red, which with the sharpened gradation of the end-point coloration permits accurate comparative observations to be made.

PROCEDURE.

Deliver 0·2 cc. of blood from a standardised capillary pipette to the bottom of a Jena or Pyrex test-tube. Wash the pipette carefully with 3 or 4 drops of distilled water contained in a small centrifuge tube and transfer the washings by means of the pipette to the Jena tube; *repeat* the washings twice. Add 0·5 cc. of aqueous silver nitrate (7·265 g. per litre; 1 cc. = 0·0025 g. NaCl), using a standardised Ostwald pipette. After mixing these, add very approximately 0·33 g. of pure powdered ammonium persulphate and wash down the adhering particles of persulphate and added liquids with 3·0 cc. of concentrated nitric acid (chlorine-free) from a pipette or a burette with ungreased tap.

Heat this mixture gently with moderate agitation and avoidance of frothing till the protein has dissolved. Clamp the test-tube in a retort stand at about 60° to the horizontal and bubble air through its contents by a fine capillary tube not necessarily of Jena glass (see Fig. 1).

Fig. 1. A. Capillary tube.
B. Short rubber tube carrying the capillary tube A in a slit.
C. Glass tube to air supply.

Bumping is entirely prevented by the stream of air bubbles, and the liquid is boiled freely for 15 or 20 seconds, using a small Bunsen burner or a larger size of microburner. The opalescence of the silver chloride precipitate is replaced by a clear greenish yellow solution containing small compact clumps or granules of silver chloride. At this stage the flame is removed and the capillary tube detached from the valvular slit in the rubber tube delivering air. The test-tube, carrying with it the capillary tube, is removed from the stand and allowed to cool.

All the samples of a series are brought to this stage and cooled thoroughly; after which air is again bubbled through by means of the capillary tube which is very readily attached and detached. 3·5 cc. of acetone are added to each

in turn from a small measuring cylinder, the air bubbles ensuring complete mixing. When more than 6 samples are in progress they should be treated with acetone and carried to the final stage in two groups; but if no more than 6, all may go through together.

When the acetone has been added to the last sample the heat of mixing will have passed off sufficiently in the first of the series, and, again connecting the air supply to its capillary tube, 0·3 cc. of concentrated ferric iron alum solution is added. A whitish precipitate usually forms due to the insolubility of the alum in acetone, and distilled water should be added drop by drop till it dissolves (usually 6 drops).

The Jena tubes are best ranged in an ordinary single row test-tube stand, with a white porcelain plate behind them and white paper beneath, using a strong front illumination from a shaded electric lamp about 6 inches away.

The first sample is then titrated against an alcoholic solution of ammonium thiocyanate from a 5 cc. microburette delivered directly into the solution by a fine capillary extension of the microburette attached by pressure tubing. The bubbles of air produce effective stirring, and the end-point is very sharp and sensitive in this illumination, especially in a darkened room. The observer's eyes should be completely protected from glare by a shade on the lamp. Titration is carried in the first sample to the earliest definite pink, and subsequent samples should be titrated by comparison to the depth of colour of the first sample.

Having completed the whole series, the samples are then transferred to tubes of colourless glass and of exactly equal bore. The solutions are carefully poured out, leaving the last few drops containing the silver chloride in the Jena tubes, and titration is then carried accurately to the colour of the first sample. Deeper solutions, if any, are corrected by titrating a solution of the same depth as the first sample to the colour of the darker solution, and subsequently subtracting the excess added from the titre of that solution. Corrections usually amount to about 0·02 cc. of alcoholic thiocyanate.

Although there are several operations, it is found in practice that they are much easier to perform than to describe and there is very little to go wrong.

Certain supplementary points in technique, which must be carefully followed.

(1) Pipettes used must be carefully re-standardised; the 0·2 cc. with mercury, as their contents are to be washed out, and the 0·5 cc. Ostwald pipettes with water as they are to be blown out after 15 seconds' drainage. The Ostwald pipettes should deliver their contents slowly, about 30 seconds as a minimum time, or wetting of the glass causes appreciable error. Iron alum is added grossly in excess of that required to combine with the thiocyanate.

(2) Dissociation is also less in acetone; therefore titration is performed, in the presence of acetone, with the minimum of water for solution of the iron alum. The standard ammonium thiocyanate cannot therefore be used in acetone solution because the alum would be precipitated by the acetone added

in the course of the titration. Dissociation is much less in alcohol than in water, though greater in alcohol than in acetone. Therefore alcoholic ammonium thiocyanate is the best practicable substitute for a solution in acetone.

(3) Ferric thiocyanate dissociates into ferric and thiocyanate ions which are colourless. The minimum dissociation takes place when there is a marked excess of either thiocyanate or iron. Therefore, to obtain the maximum coloration with the minimum of thiocyanate excess, a large quantity of iron must be added.

(4) Very occasionally a sample turns pink 0·1 or 0·2 cc. before the true end-point—especially if the thiocyanate standard has been run in too quickly. These samples always fade completely before the final accurate colour comparison is made and no gross error results when the final correction is added.

(5) Brownish colours in the mixture before titration are due to incomplete cooling before or after addition of acetone. If samples are allowed to stand this colour disappears in half-an-hour.

Results of 9 consecutive observations on whole blood.

Total chloride per 100 cc.	Known chloride added per 100 cc.	Chloride of original blood sample
g.	g.	g.
0·429	0·000	0·429*
0·446	0·025	0·421
0·424	0·000	0·424
0·468	0·044	0·424
0·519	0·094	0·425
0·423	0·000	0·423
0·452	0·031	0·421
0·423	0·000	0·423
0·517	0·094	0·423

Results of 9 consecutive observations on serum.

Total chloride per 100 cc.	Known chloride added per 100 cc.	Chloride of original serum sample
g.	g.	g.
0·553	0·000	0·553
0·581	0·025	0·556
0·553	0·000	0·553
0·602	0·044	0·558
0·557	0·000	0·557
0·653	0·094	0·559
0·558	0·000	0·558
0·574	0·025	0·549*
0·596	0·038	0·558

* No known cause for this deviation. Rehberg claims errors of only 2 or 3 mg. in his estimations of serum chloride, using 0·1 cc. of serum. This amounts to an error of $\frac{1}{3}$ or $\frac{1}{2}$ %. Such results have frequently been obtained on a short series of estimations. Errors in the second figure are very rare. The published series contain the error due to pipette calibration since a different pipette was used for each sample. A closer approximation of results can be obtained by using the same pipette throughout.

SUMMARY.

(1) A simple and rapid method of estimating blood chlorides on 0·2 cc. has been described.

(2) Using whole blood the outside error is usually 4 milligrams of NaCl per 100 cc. of blood, with a similar percentage error for serum.

(3) Larger errors occur for unknown reasons in about one-tenth of the samples, but remain in the third place of decimals.

I wish to thank Prof. Raper and Prof. Craven Moore for their interest in this work, and Dr Roberts for testing the accuracy of the method.

REFERENCES.

Claudius (1924). *Acta Medica Scand.* **61**, 3.
Fowweather (1926). *Biochem. J.* **20**, 93.
Rehberg (1925). *Biochem. J.* **19**, 270.

[Note added February 4th, 1927]. Although the method of titration already described, followed by correction in comparator tubes, is the better for occasional use and for those workers whose colour sense is not acute, a modification has been introduced since going to press, which yields better results in the hands of practised workers whose colour perception is good. The acetone is added to the cooled digested samples, taking not more than two or three of a series at once. The mixture is then cooled for about 30 seconds and titrated directly with ammonium thiocyanate. The thiocyanate is run in at a moderate rate and a *brisk* stream of air bubbles is passed to ensure *rapid* mixing. Titration is complete at the first distinct uniform red flush which appears throughout the solution.

Results in duplicate by this method compare very closely. The error is usually about 1 to 3 milligrams, occasionally 6 or 8 milligrams, in the serum and whole blood and about 6 or 8 milligrams in the corpuscles. Larger errors than 8 milligrams are rare in the corpuscles provided that the corpuscular mass has been thoroughly mixed.

The time of estimation is reduced by this modification to an average of 9 or 10 minutes per sample over a series, and 13 minutes for a single estimation.

VI. THE MICRO-ESTIMATION OF IRON IN BLOOD.

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(*Received November 15th, 1926.*)

The estimation of iron in small quantities of blood is almost universally colorimetric. The thiocyanate colour is preferable to the ferrocyanide because of its greater intensity and the unsuitability of blue solutions for colorimetric work.

The use of acetone to intensify colour and minimise the influence of interfering substances was introduced by Mariott and Wolf [1906] and used by Fowweather [1926]. It was not used by Walker [1925] and was criticised by Wong [1923] who considered that its evaporation introduced considerable errors. Personal observations strongly confirm Fowweather's contention that this error is negligible and entirely outweighed by the advantages outlined by Mariott and Wolf.

Destruction of organic matter has been accomplished by concentrated hydrobromic acid [Berman, 1918] and by potassium chlorate [Wong, 1923], and recently Fowweather has published another method using Wong's procedure, but replacing the potassium chlorate by perhydrol (100 volume H_2O_2) in order to prevent the violent bumping and frequent loss of samples with potassium chlorate.

Using Fowweather's method occasional bumping and loss of samples was still troublesome. With different samples of perhydrol, oxidation was found to be somewhat tedious. It appeared that his order of accuracy would have been attained with practice. The following has been elaborated as a shorter method for a series of estimations, entirely without risk of bumping and with probably a greater accuracy than that claimed by Fowweather.

The method depends on the rapid oxidation of blood proteins by ammonium persulphate and nitric acid, with subsequent colorimetric estimation of iron as thiocyanate in the presence of acetone, against an artificial colour standard. This enables a series of estimations to be made using the same standard whereas alternatively for accurate working it becomes necessary to make an iron standard for each sample.

PROCEDURE.

0·2 cc. of blood is delivered from a standardised pipette to the bottom of a 4½ or 5 inch Jena or Pyrex test-tube. The pipette is washed by sucking two or three drops of water up and down the pipette from a small centrifuge tube and then transferring these by the pipette to the Jena tube. The process of washing is repeated twice.

0·33 g. of a well mixed sample of powdered ammonium persulphate is added to the contents and washed down to the bottom of the tube with 2 cc. of concentrated nitric acid.

The mixture is gently heated with moderate shaking till the protein is dissolved and the solution is clear. The tube is clamped in a retort stand and heating is continued for about 1 minute, air being bubbled through the solution from a fine capillary tube in order to prevent bumping. The solution darkens slightly in shade and then grows lighter again. This may be taken as an index of complete oxidation though the slight colour change is not always noted. The flame is withdrawn and 6 cc. of distilled water are added from a burette, the stream of air ensuring mixing. The capillary tube is then withdrawn and inserted without washing into the next of the series of samples.

The first sample, which has now been boiled and diluted with 6 cc. of water, is poured into a 50 cc. flask and the test-tube washed out three times with 2 cc. of distilled water from a burette, each time adding the washings to the 50 cc. flask. The test-tube is then placed on clean blotting paper by the flask ready for the final washing with acetone.

When all the samples are dissolved and delivered into 50 cc. flasks, 25 cc. of acetone are taken in a small measuring cylinder and the first of the series of test-tubes which has already been washed with distilled water is washed out again with the acetone, the whole 25 cc. of acetone washings being delivered to the first flask which is then gently shaken and immersed in cold water for 5 minutes. 5 cc. of concentrated ammonium thiocyanate solution (24 g. per 100 cc. of water) are added and the volume is made up to the 50 cc. mark with distilled water. The iron estimation is then made colorimetrically against an artificial standard, consisting of a mixture of cochineal, methyl red, and hydrochloric acid dissolved in an equal mixture of acetone and water. The proportions are as follows:

4 cc. cochineal solution (British Drug Houses' Indicator).
3·2 cc. methyl red solution (British Drug Houses' Indicator).
4 cc. dilute hydrochloric acid (1 in 5).

This is diluted with equal parts of acetone and water to the approximate depth of colour required and a final adjustment of tint is made by adding drops of methyl red if the orange tint predominates, and of cochineal solution if the red is too clear. The colour is permanent and indistinguishable from that of the thiocyanate.

Before the ammonium thiocyanate is added to the first of a series of estimations it is best to add the acetone washings to the second of the series so that this may be cooling while the colorimetric readings are taken on the first sample.

Finally 1 cc. of a solution containing 0·1 g. of ferric iron per litre (*v.* Fowweather) is added from an Ostwald pipette to a Jena tube. 0·33 g. of ammonium persulphate and 2 cc. of concentrated nitric acid are also introduced and these are boiled down to about 2 cc. as are the samples containing blood. The solution is made up to 50 cc. as already described and compared with the artificial standard. A blood sample of equal tint to this iron sample contains 50 mg. of iron per 100 cc. of blood.

In explanation it is well to point out that ammonium persulphate as supplied by manufacturers contains minute traces of iron. Hence a well-mixed sample must be used and the error balanced by a similar addition to the iron standard. Iron-free nitric acid also gives a slight coloration with thiocyanate which is corrected by the nitric acid in the standard.

Practical precautions.

(1) Slight differences in the opacity of solutions always cause trouble in colorimetric work. These can be corrected by increasing the illumination on the opaque side.

(2) Bubbles of gas are set free when acetone is mixed with water. The solutions should be shaken and these allowed to escape or there will be great difficulty with the colorimeter.

(3) The colorimeter is the main source of error. When the colorimetric readings are close and the results divergent the probability is that the illumination has been unequal. The illumination is best adjusted between each reading. 15 mm. is the best level for most people when the iron content is normal, and 20 mm. for anaemic bloods. The personal factor largely governs other optimal conditions for any one observer.

Results.

Results of 18 *consecutive observations, made with two different samples of human blood A and B.*

Sample A of blood	Total estimated iron in sample mg.	Known added iron mg.	Total iron minus added iron = iron in 0·2 cc. of blood taken mg.
1	0·109	0·004	0·105
2	0·106	0·000	0·106
3*	0·108	0·006	0·102
4	0·110	0·003	0·107
5	0·107	0·000	0·107
6	0·112	0·005	0·107

* In the case of this sample the acetone was delivered straight to the flask instead of using it for the final washing of the test-tube. This represented a loss of 0·004 mg. of iron.

Sample B of blood	Total estimated iron in sample mg.	Known added iron mg.	Total iron minus added iron = iron in 0·2 cc. of blood taken mg.
1	0·103	0·008	0·095
2	0·098	0·003	0·095
3	0·095	0·000	0·095
4	0·103	0·006	0·097
5	0·098	0·000	0·098
6	0·099	0·004	0·095
7	0·102	0·005	0·097
8	0·096	0·000	0·096
9	0·105	0·008	0·097
10	0·099	0·002	0·097
11	0·096	0·000	0·096
12	0·103	0·007	0·096

All results are stated to the nearest significant figure.

Summary.

A simple and rapid method for the accurate estimation of blood-iron using 0·2 cc. of blood is described. The method depends on a colorimetric comparison of the iron as ferric thiocyanate with an artificial colour standard.

My thanks are due to Prof. Raper and Prof. Craven Moore for their kind interest in this work and to Dr F. M. Irvine for one or two chemical suggestions.

REFERENCES.

Berman (1918). *J. Biol. Chem.* **35**, 231.
Fowweather (1926). *Biochem. J.* **20**, 93.
Mariott and Wolf (1906). *J. Biol. Chem.* **1**, 456.
Walker (1925). *Analyst*, **50**, 279.
Wong (1923). *J. Biol. Chem.* **55**, 421.

VII. STUDIES IN CARBOHYDRATE METABOLISM.

II. INFLUENCE OF METHYLGLYOXAL AND OTHER POSSIBLE INTERMEDIARIES UPON INSULIN HYPOGLYCAEMIA.

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(*Received November 17th, 1926.*)

In a previous communication [Kermack, Lambie and Slater, 1926], it has been shown that dihydroxyacetone when administered subcutaneously causes rabbits and mice to recover from insulin hypoglycaemia. It seemed to us that no significant difference could be discovered between the time taken for recovery after injection of dihydroxyacetone as compared with that taken after injection of glucose. In a paper by Campbell and Hepburn [1926], it has likewise been demonstrated that dihydroxyacetone causes prompt recovery from the symptoms of insulin poisoning, but these workers are of the opinion that glucose effects recovery more rapidly than does dihydroxyacetone, at least when both substances are injected intravenously. On the other hand, Hewitt and Reeves [1926], who have also confirmed the fact that dihydroxyacetone cures insulin hypoglycaemia, state that after administration of dihydroxyacetone recovery takes place in a manner identical with that seen when glucose is injected. They, like ourselves, injected the carbohydrate subcutaneously. We have now carried out further experiments to discover whether the discrepancy between our results together with those of Hewitt and Reeves on the one hand, and those of Campbell and Hepburn on the other, was due to the method of administration. We have not been able to convince ourselves that with intravenous injection there is any significant difference in the time taken for recovery. Naturally, without using very large numbers of animals it would be impossible to determine this point conclusively owing to the varying response of different animals or even of the same animal at different times.

In the paper by Hewitt and Reeves, referred to above, it is shown that glyceric aldehyde is not able to effect recovery from insulin hypoglycaemia.

We have prepared methylglyoxal by a method described by Fischer and Taube [1924, 1926] and we have tested this substance in a similar way. Briefly, we may say that the results were entirely negative and we even

received the impression that the substance possessed some toxicity. In all, seven animals were tested and in none of them did recovery take place. In each case the substance was injected subcutaneously, and usually several doses were administered. The rabbits were allowed to become completely comatose before injection of the test substance in order to minimise the risk of fallacies due to spontaneous recovery.

The following is a typical experiment.

Rabbit, female, weight 2320 g., starved 24 hours:

12.0 noon. 10 units insulin per kg.
2.50 p.m. Convulsions followed by recovery.
3.45 ,, Convulsions with imperfect recovery.
4.0 ,, Convulsions and coma.
4.18 ,, Methylglyoxal 0·7 g. per kg. subcutaneously.
4.21 ,, Violent convulsions.
4.32 ,, Sprawling.
4.41 ,, Convulsion, deeply comatose.
4.44 ,, 0·7 g. methylglyoxal per kg.
4.48 ,, Deeply comatose, lying on side apparently moribund, rapid respiration.
4.55 ,, Violent convulsion.
5.5 ,, Dihydroxyacetone 0·7 g. per kg. subcutaneously.
5.10 ,, Sitting up in normal position.

Recovery complete and permanent.

Three of the animals to which large quantities of methylglyoxal were given, failed to recover even after subsequent injection of glucose or dihydroxyacetone. It is because of this and the peculiar deep breathing observed in these hypoglycaemic rabbits after repeated large doses of methylglyoxal, that we are led to believe that it possesses some toxic action.

A note may be added here on the preparation of methylglyoxal from dihydroxyacetone.

Experiments carried out according to the instructions of Fischer and Taube in which dihydroxyacetone (5·0 g.) and phosphorus pentoxide (15·0 g.) were thoroughly mixed and very gently heated until the reaction proceeded spontaneously, the product being condensed by liquid air or by a good freezing mixture, did not give yields as good as those claimed by these authors. The product, the yield of which varied from 1–2 g., polymerised very rapidly and became insoluble in water and so had to be mixed with an equal volume of water very soon after the distillation. If the quantity of phosphorus pentoxide was reduced, the yield became greater (up to 2·6 g.), but the product obtained was less pure, as shown by the yield of di-semicarbazone (M.P. 253°), obtained from a weighed quantity.

For instance, the products from two experiments using the larger quantity of phosphorus pentoxide gave 96 % and 99 % respectively of the theoretical yield of semicarbazone, whilst with the smaller quantity only 60·5 % of the

theoretical yield was obtained. The material obtained by the use of the smaller quantity of phosphorus pentoxide did not become vitreous as the result of polymerisation as did the other, but only thickened to a very viscous consistency.

Since either dihydroxyacetone or methylglyoxal might conceivably form lactic acid in the animal body, while the latter might either undergo complete combustion or form glucose, it was considered of interest to investigate further the influence of this acid upon insulin hypoglycaemia. We entirely failed to effect recovery or to prevent convulsions, either by subcutaneous or by intravenous injection of the substance. Our experiments therefore confirm the impression gained by Noble and Macleod [1923] who, while not coming to any final conclusion, did not obtain evidence of recovery.

Glycerol also failed to produce recovery or to prevent convulsions, even when large quantities were injected intravenously. This agrees with the results of Noble and Macleod [1923], although it should be noted that Voegtlin, Dunn and Thompson [1924, 1925], claim that glycerol administered intraperitoneally or by mouth protects mice from insulin hypoglycaemia and causes a rise in the blood-sugar of fasting rabbits. If it is active at all, it is certainly, in our experience, in no way comparable to either dihydroxyacetone or glucose.

Sodium pyruvate, like methylglyoxal, so far from removing the symptoms of hypoglycaemia, appeared to have a distinct toxic action, which, in some animals interfered with the recovery by glucose or dihydroxyacetone.

During the present work, sodium citrate and rhamnose were also tested, in each case with negative results.

Discussion.

From the above, it is evident that there can be no comparison between dihydroxyacetone and any of the other assumed intermediaries in carbohydrate metabolism containing three carbon atoms, namely, methylglyoxal, glyceric aldehyde, glycerol, lactic acid and sodium pyruvate.

In our previous communication we left it an open question as to whether dihydroxyacetone acted by being converted into glucose or whether it could be directly oxidised, although we came to the conclusion that the balance of evidence was in favour of the latter assumption. Since this communication was written, a paper has appeared by Campbell, Fletcher, Hepburn and Markowitz [1926] in which the conclusion is reached, from a number of different lines of investigation, that dihydroxyacetone is apparently completely converted into glucose as the first step in its metabolism in the animal body. Nevertheless, we consider that such a definite conclusion is not justified. There is, we think, a considerable body of evidence to show that dihydroxyacetone may be metabolised more rapidly than glucose. Firstly, the blood-sugar curves reported by Isaac and Adler [1924], Rabinowitch [1925, 1, 2], Mason [1926, 1] and ourselves all indicate that dihydroxyacetone does not

cause an increase in the blood-sugar comparable to that produced by an equal amount of glucose. Even Campbell [1926] found very little rise in the blood-sugar curve after dihydroxyacetone although, curiously, he got very little rise in his control experiments with glucose. Secondly, Isaac and Adler [1924] have brought evidence to show that dihydroxyacetone can form glycogen with greater ease than can glucose. Thirdly, the respiratory metabolism experiments of Mason [1926, 2] indicate that dihydroxyacetone is oxidised more easily and more completely than is glucose in the normal individual and to a less extent in the diabetic[1]. Fourthly, in the perfusion experiment upon the eviscerated and decerebrate cat reported in our first communication, it was shown that dihydroxyacetone disappears from the blood more rapidly than glucose when perfused at the same rate, even although the liver is out of the circulation.

The problem is a difficult one, but it is not easy to reconcile these observations with the conclusions reached by Campbell, Fletcher, Hepburn and Markowitz. Particularly when we consider the greater ease with which dihydroxyacetone can be oxidised *in vitro* as compared with glucose does it seem probable that it can undergo, to some extent at least, direct oxidation. Campbell, Fletcher *et al.* found that in the completely depancreatised dog dihydroxyacetone was not only converted into glucose and quantitatively excreted as such, but that the elimination was as rapid as if an equivalent quantity of glucose had been administered. We have examined two cases of complete diabetes in the human subject, that is to say, patients who were excreting more sugar than they were taking in. In such clinical experiments it is difficult to obtain clear cut results but in general our conclusion was that the dihydroxyacetone administered was almost quantitatively excreted as glucose. Incidentally, it was confirmed by polariscopic examination that the reducing substance in the urine was actually dextrose.

It seems then that in extreme diabetes dihydroxyacetone may be converted rapidly into glucose, whereas, where insulin is available, it is more easily utilised than glucose. This conclusion is confirmed by the observations of Mason [1926, 2], who found that in diabetics dihydroxyacetone did not exhibit as great a difference in its rate of metabolism when compared with glucose as was found in the case of normal individuals.

In order to explain this result we venture to suggest the hypothesis that an equilibrium exists in the body between dihydroxyacetone and glucose (similar to that which appears to exist *in vitro* in weak alkaline solution), such that in diabetes or in the absence of insulin the tendency is in the direction dihydroxyacetone to glucose and in the opposite direction when there are inadequate supplies of insulin. Administration of excess of dihydroxy-

[1] [Note added January 1, 1927.] Since the present paper was submitted for publication, the results of Mason have been amply confirmed by one of us (C. G. L.) and it has further been shown, by intravenous injection, that greater and more rapid increase in metabolism after dihydroxyacetone, as compared with glucose, is not due to its more rapid absorption. These results will be published fully elsewhere.

acetone, even in the presence of insulin, would by mass action give rise to a transient hyperglycaemia.

Dihydroxyacetone will naturally be in equilibrium with glycerol and with methylglyoxal, and through the latter with lactic acid and alanine. If dihydroxyacetone is being rapidly converted into glucose, as in the diabetic organism, not only will all these substances tend to produce glucose, but also compounds such as propionic acid and fatty acids containing an odd number of carbon atoms as well as certain amino-acids which ultimately on degradation give rise to fragments containing three carbon atoms. On the other hand, after administration of dihydroxyacetone, an increase in the excretion of lactic acid is to be expected, as this is a comparatively stable product in equilibrium with dihydroxyacetone through methylglyoxal, and it has been shown, in fact, by Isaac and Adler [1924] and by Mason [1926, 1, 2] that there is an increase in the production of lactic acid under these conditions. Again, it has been shown by Smedley MacLean and Hoffert [1926] that in yeast the probability is that fat is produced directly from at least three glucose molecules and, if this applies to the animal organism, the tendency often observed in early diabetics to the deposition of fat would be simply due to the mass effect of the large amount of glucose formed and its non-conversion into dihydroxyacetone. Naturally, the fat deposits disappear when the organism becomes increasingly dependent upon these stores of fat as a source of energy. The glycerol formed from their catabolism would then go through dihydroxyacetone into glucose and, of course, it is well known that glycerol yields glucose quantitatively in the complete diabetic.

It at first seems an important objection to this view that dihydroxyacetone exists, if it exists at all, in the blood stream in a concentration of at any rate less than 1 in 20,000 (human), to 1 in 10,000 (rabbit). However, if dihydroxyacetone is extraordinarily easily oxidised and if the conversion of glucose into dihydroxyacetone under the influence of insulin only takes place comparatively slowly as compared with the rate of oxidation of dihydroxyacetone, and further when it is remembered that the site of the action of insulin is not in the blood but in the tissues [Eadie, Macleod and Noble, 1923; Burn and Dale, 1925; Lambie, 1926], such a low concentration of dihydroxyacetone in the blood stream is only to be expected.

If the above hypothesis were true, the effect of dihydroxyacetone in causing animals to recover from insulin hypoglycaemia would be that it is directly oxidised, thereby supplying energy for the activity of the nerve cells. The absence of effect in the case of any of the other supposed intermediaries would be due to the fact that they can neither be themselves oxidised in such a way that their energy can be utilised in cellular metabolism nor converted into dihydroxyacetone at a sufficient rate. Presumably the only substances which could be so converted would be glucose, fructose and the few substances which cause recovery from insulin hypoglycaemia.

That glucose and dihydroxyacetone appear to effect recovery in the same

time may be due to the fact that the limiting factor is not simply the rate at which a sufficient quantity of dihydroxyacetone is brought in contact with the tissues, whether from glucose or directly, but the time taken for the nerve and muscle cells to recover from the secondary effects of the hypoglycaemic state.

Summary.

(1) No significant difference could be observed in the time taken to recover from insulin hypoglycaemia following intravenous dihydroxyacetone administration as compared with glucose.

(2) Methylglyoxal and sodium pyruvate fail to cause animals to recover from insulin hypoglycaemia and appear to have a toxic action.

(3) Negative results were also obtained with sodium lactate, glycerol, sodium citrate and rhamnose.

REFERENCES.

Burn and Dale (1925). *J. Physiol.* **59**, 164.
Campbell (1926). *J. Biol. Chem.* **67**, 59.
Campbell, Fletcher, Hepburn and Markowitz (1926). *J. Biol. Chem.* **67**, lvii.
Campbell and Hepburn (1926). *J. Biol. Chem.* **68**, 575.
Eadie, Macleod and Noble (1923). *Amer. J. Physiol.* **65**, 462.
Fischer and Taube (1924). *Ber. deutsch. chem. Ges.* **57**, 1502.
—— —— (1926). *Ber. deutsch. chem. Ges.* **59**, 857.
Hewitt and Reeves (1926). *Lancet*, i, 703.
Isaac and Adler (1924). *Klin. Woch.* **3**, 1208.
Kermack, Lambie and Slater (1926). *Biochem. J.* **20**, 486.
Lambie (1926). *Brit. J. Exp. Path.* **7**, 22.
Mason (1926, 1). *Canad. Med. Assoc. J.* **16**, 367.
—— (1926, 2). *J. Clin. Invest.* **2**, 521, 533.
Noble and Macleod (1923). *Amer. J. Physiol.* **64**, 547.
Rabinowitch (1925, 1). *J. Biol. Chem.* **65**, 55.
—— (1925, 2). *Canad. Med. Assoc. J.* **15**, 374.
Smedley MacLean and Hoffert (1926). *Biochem. J.* **20**, 343.
Voegtlin, Dunn and Thompson (1924). *Amer. J. Physiol.* **71**, 574.
Voegtlin, Thompson and Dunn (1925). *J. Biol. Chem.* **64**, 639.

VIII. THE HEAT-DENATURATION OF PROTEINS. PART IV.

THE FREE BASIC AND ACIDIC GROUPS OF FRESH AND DENATURED HAEMOGLOBIN.

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(*Received November 22nd, 1926.*)

The present paper[1] is an account of an attempt to determine directly by titration, whether any change in the total number of free basic and acidic groups takes place in the protein molecule on denaturation.

The various views of the chemical process underlying heat-denaturation may be said to lie between two extremes which are absolutely opposed. Robertson [1918] is of the opinion that this fundamental chemical reaction is one of condensation of carboxyl and amino-groups of contiguous molecules, while a further group of workers, notably Lepeschkin [1922] and Wu and Wu [1925] prefer to regard it as a mild hydrolysis.

Consequently titration, if it can be rendered sufficiently accurate, should afford a crucial test of these theories. On the view of Robertson the total number of basic and acidic groups should be diminished by denaturation; while on the opposed view this number should either increase or remain constant according to whether the linkages hydrolysed give rise to such groups, or whether, as Lepeschkin thinks, the change is more comparable with the hydrolysis of sugars.

Harris [1923, 1] in the course of an investigation on a hitherto undiscovered sulphur linking in proteins, carried out formaldehyde titrations on fresh and denatured egg-albumin and came to the conclusion that no detectable change occurred on denaturation.

It may be noted in this connection that Sørensen and Jurgensen [1910] showed that on continued heating of egg-albumin (up to 24 hours in a boiling water-bath) the formaldehyde titre of the solution, which falls at first owing to the removal of protein, increases towards the end of the reaction owing, they conclude, to hydrolysis followed by re-solution of the fragments resulting from the cleavage. This reaction is of course subsequent to denaturation. In the course of the same paper they say that they do not consider that any

[1] For the earlier papers of this series cf. Lewis [1926].

disintegration of the protein takes place on simple denaturation. The workers mentioned above as being of the opinion that heat-denaturation is a hydrolysis do not appear to have in mind such a violent reaction as that with which Sørensen and Jurgensen dealt.

In the present work the titratable amino-groups of the haemoglobin molecule have been determined before and after heat-denaturation. No change is indicated.

Investigation of the acidic groups of the molecule before and after denaturation by heat indicates that the reaction causes no detectable change in the number of acidic groups.

Section 1. The free amino-groups of fresh and denatured haemoglobin.

The method employed here was that used by Harris [1923, 2; 1924, 1, 2] for the titration of various amino-acids and complex ampholytes.

The method is to determine the p_H reached by the ampholyte solution on the addition of various amounts of standard acid. The same treatment is accorded to an equal bulk of the solvent used. The curve obtained in this latter case affords a blank correction curve from which it is possible to determine the amount of acid necessary to bring the solvent to any particular p_H. This is then subtracted from the amount of acid necessary to bring the ampholyte solution to the same p_H. The resulting figure is the amount of acid taken up by the ampholyte itself in moving to this p_H and is termed the "corrected titre" of the ampholyte. When these corrected titres are plotted against p_H they yield an S curve showing two regions in which it is asymptotic to the p_H axis. The first is in the initial stages of the titration and the second at the end of the titration where the curve becomes asymptotic to some particular titre. This latter value is the actual titre of the ampholyte, and is the amount of acid necessary to neutralise its basic groups.

Both with the solution and the solvent the titration is commenced from neutrality. The curve of corrected titres against p_H is termed the "titration curve" of the ampholyte.

Experimental.

In the following work each point was determined separately. That is to say that the whole titration was not carried through with one solution but a separate solution was made up for each amount of acid added. This was deemed preferable as in this manner the haemoglobin was only under the influence of the acid for 5 to 10 minutes during each determination. This tended to eliminate error due to possible denaturation by the acid.

The solutions were made up by taking 20 cc. of 1 % fresh haemoglobin each time. This was either denatured or retained as such. The denaturing was carried out by immersing a corked flask, containing the haemoglobin, in the steam from a briskly boiling water-bath for 10 minutes. The flask and

contents were subsequently cooled and every drop of condensed moisture in the flask collected back into the original liquid. A little loss of water would be bound to ensue but it would be relatively negligible. To the solutions so obtained the desired amount of acid was then added.

The p_H of each solution was then determined at 25° by means of the quinhydrone electrode, by measurement of the E.M.F. of a cell of the type:

Gold electrode	Solution under test saturated with quinhydrone	Saturated KCl	Normal calomel electrode

The solution contained 1 g. of haemoglobin and 0·054 g. of ammonium sulphate per 100 cc. The titration was performed with 0·096 *N* H_2SO_4. The titrations are accurate to ± 0·01 cc.

The following results were obtained. For the sake of brevity only mean p_H values are given. Each value is the mean of six independent readings, the accuracy being ± 0·003 p_H.

Table I. *Titration of* 20 *cc. of* 0·054 % $(NH_4)_2SO_4$ *with* 0·096 N H_2SO_4.

cc. H_2SO_4	0	0·02	0·03	0·05	0·20	0·30	0·50	1·00	1·50	2·00	2·50	3·00
p_H	6·771	4·117	3·802	3·564	3·107	2·936	2·724	2·462	2·301	2·195	2·112	2·039

From Table I a correction curve was drawn from which the values in the horizontal rows headed "blank" of the tables below are obtained.

Table II. *The titration of* 20 *cc. of* 1 % *fresh* (*undenatured*) *haemoglobin containing* 0·054 % *of* $(NH_4)_2SO_4$, *with* 0·096 N H_2SO_4 *at* 25°.

cc. H_2SO_4	0	0·30	1·00	2·00	3·00	4·00	5·00	6·00
p_H	6·781	5·521	4·451	3·440	2·809	2·443	2·198	2·047
Blank in cc.	0	0·015	0·02	0·075	0·32	1·08	2·00	2·98
Corrected titre in cc.	0	0·285	0·98	1·925	2·68	2·92	3·00	3·02

Table III. *The titration of* 20 *cc.* 1 % *denatured haemoglobin containing* 0·054 % *of* $(NH_4)_2SO_4$, *with* 0·096 N H_2SO_4 *at* 25°.

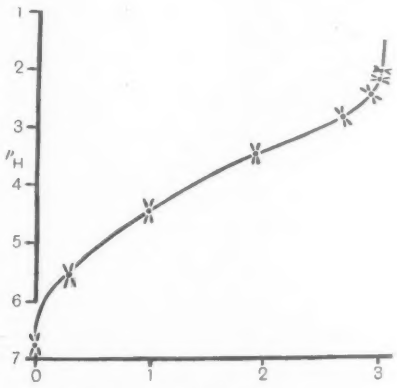
cc. H_2SO_4	0	0·30	1·00	2·00	3·00	4·00	5·00	6·00
p_H	6·778	5·529	4·435	3·441	2·812	2·433	2·195	2·047
Blank in cc.	0	0·015	0·02	0·075	0·31	1·09	2·00	2·98
Corrected titre in cc.	0	0·285	0·98	1·925	2·69	2·91	3·00	3·02

The titration curves derived from these tables are shown in Fig. 1. These curves show the variation of corrected titre with p_H.

It will be seen that the titration curves of fresh and denatured haemoglobin are sensibly identical. Either corresponds to a titration of 3·05 cc. of 0·096 *N* H_2SO_4 for 20 cc. of 1 % haemoglobin. From this it follows that 100 g. of haemoglobin contain 2·05 g. of titratable nitrogen.

It is generally assumed that the α-amino-groups of amino-acids are entirely utilised in linkages in the protein molecule. Consequently the titratable basic groups will be the second basic groups of the dibasic acids in the molecule. According to Van Slyke and Birchard [1913] the diamino-acids present in haemoglobin are lysine, histidine and arginine which account for 11·90, 12·69

and 7·72 % respectively of the total nitrogen of the molecule. Under the present circumstances one half of the total lysine nitrogen, one-third of the histidine nitrogen and one-quarter of the arginine nitrogen[1] will be titratable; *i.e.* (5·45 + 4·23 + 1·93) = 11·61 % of the total nitrogen of the molecule. The total nitrogen of the haemoglobin molecule of the ox being given by Mathews [1924] as 17·70 % of the molecule, it would be expected on this basis that there would be 2·055 g. of titratable nitrogen per 100 g. of haemoglobin. This is in very good agreement with the value obtained above. It is stated with some degree of certainty that the present results point to the absence of change in the number of titratable basic groups of the haemoglobin molecule on denaturation.



Titre in cc. of 0·096 *N* H_2SO_4.

Fig. 1. Corrected titration curves of fresh and denatured haemoglobin.
• Titration of fresh haemoglobin.
X Titration of denatured haemoglobin.

Assuming the value of 16,660 as the equivalent weight of haemoglobin [Mathews, 1924] it is found that these figures correspond to 24·4 titratable nitrogen atoms per equivalent.

Section 2. The free carboxyl and other acidic groups of fresh and denatured haemoglobin.

In order to investigate this problem, use was made of a modification of the formaldehyde titration of Sørensen. Normally a titration of carboxyl groups has to be carried to a very alkaline end-point to obtain complete titration. In this case the correction for the dilution of the system of titrant

[1] Lysine, histidine and arginine contain respectively 2, 3 and 4 nitrogen atoms, of which two are titratable in each case. In lysine and arginine these two atoms are members of amino-groups and in histidine one is a member of an iminazole ring. In each case one of these two basic groups is an α-amino-group and therefore assumed bound. This leaves one nitrogen atom titratable in each of these amino-acids. Hence the above fractions.

becomes undesirably large. If, however, formaldehyde be added to the protein before titration the acidic nature of the protein is much enhanced and the titration is complete at about p_H 9. This is explained [see Harris, 1924, 2] as being due to the formation of methylene-imino-derivatives of the amino-acids present

$$(HOOC.R.NH_2 + HCHO \rightarrow HOOC.R.N = CH_2 + H.OH),$$

which have an acidic dissociation constant approximately one thousand times that of the original protein. The addition of formaldehyde therefore forms a means of enhancing the accuracy of the titration.

Harris [1924, 2] also advocates the use of alcohol to enhance the accuracy of the titration, but this is not permissible in the present instance owing to the action of alcohol on the protein itself, which reaction is identified with heat-denaturation by Robertson.

If it is desired to perform a titration of the protein and to form an accurate estimate of the total number of acidic groups of the molecule, it is necessary to determine the p_H of successive solutions containing protein, formaldehyde, and different known amounts of alkali. Three methods of making these determinations suggest themselves, namely, the hydrogen gas electrode, the Donnan-Allmand electrode and the glass electrode. In this case the quin-hydrone electrode may not be used on account of the alkalinity of the solutions near the end-point.

Of these electrodes the Donnan-Allmand electrode was first utilised. It was not desirable that the oxyhaemoglobin should be exposed to the reducing action of the hydrogen.

The results obtained using the Donnan-Allmand electrode indicated that denatured protein binds more alkali than does fresh. In other words, that by the process of heat denaturation fresh acidic groups were uncovered. The difference, however, was but slight and the results appeared unsatisfactory. The reproducibility was poor. Furthermore the electrodes required periods up to 48 hours to come to equilibrium. In work of the present nature it is most undesirable that the protein should be exposed to the alkali for this length of time. It is almost inevitable that some hydrolytic decomposition, subsequent to denaturation proper, should have taken place during this period.

Consequently a similar series of determinations was made with the glass electrode. The method of p_H measurement adopted was that described by Mrs Kerridge [1925]. As in dealing with the glass electrode it is required to measure the E.M.F. of a cell of very high resistance, the ordinary galvanometric method is not feasible. Instead a system is substituted in which the detector is a quadrant electrometer. The electrometer used was a Lindemann electrometer as described by Lindemann and Keeley [1924]. The greatest accuracy obtained is 0·4 to 0·5 millivolt. While this may be capable of improvement the objection still remains that any electrostatic method is extraordinarily sensitive to stray fields of force and to possible leakages of charge. It was found necessary to work within a hollow earthed conductor, and to take the

greatest care that all apparatus was well insulated. As insulating medium amberite was utilised as suggested by Mrs Kerridge. Even so leakage of charge was apt to become a troublesome factor in wet weather. On the whole, however, this seems to be the most satisfactory method of measuring the p_H of alkaline solutions. Its two great advantages are rapidity and a sensible absence of diffusion.

The solutions were made up by taking 20 cc. of 2 % fresh haemoglobin each time. This was either denatured or retained as such. To this solution 8 cc. of 10 % formaldehyde, previously adjusted to p_H 7·0, were added. Following this, the known amount of NaOH was added and the p_H determined. The results are given below. The "blank" determinations are not recorded as they have no bearing on the point at issue.

Here again, for the sake of brevity, detailed readings are omitted. The readings in Table IV are the mean of five readings in each case with independent solutions. The titres are accurate to 0·01 cc. The p_H measurements are accurate to $\pm$ 0·006 p_H.

Table IV. *Formaldehyde titration of* 20 *cc.* 2·0 % *haemoglobin containing* 0·3 % $(NH_4)_2SO_4$ *with* 8 *cc. formaldehyde added. Temperature* 18°. *Titrant* 0·360 N *NaOH.*

Titre		0	0·20	0·40	0·60	1·00	1·20	1·60	2·00	2·60	3·00
p_H of haemoglobin	Fresh	7·18	7·66	7·98	8·25	8·66	8·81	9·15	9·50	10·05	10·38
	Denatured	7·18	7·66	7·97	8·25	8·66	8·80	9·15	9·50	10·05	10·38

From the above figures it will be seen that there is no discernible difference between the fresh and denatured material.

These results are not so accurate as those of Section 1. On the present results the writer is only prepared to state that if there is a change in the number of free acidic groups in the molecule it is too small to be detected, at least by the present means. Taken in conjunction with the results of Section 1 it becomes quite probable that actually there is no change on denaturation.

The present results are definitely opposed to the view adopted by Robertson [1918], namely that denaturation entails the chemical condensation of free amino-groups in adjacent molecules.

Lepeschkin [1922] has suggested that the reaction involved is a hydrolysis of the same nature as that by which a polysaccharide is broken up into its constituent units. The results embodied in the present paper point to a grouping of some such character being involved. Certainly the linkage involved cannot be the peptide link (—CONH—) which, on *a priori* grounds might have been thought important for such a reaction.

It is of interest here to record a titration in full and thus to determine the number of free acidic groups in one equivalent weight of haemoglobin. The necessary blank measurements have been made and the corrected titration curve drawn as shown in Fig. 2.

The measurements are recorded below.

Table V. *Titration of* 20 *cc.* 0·3 % *ammonium sulphate solution to which had been added* 8 *cc. of* 10 % *formaldehyde. Titrant* 0·360 N *NaOH.*

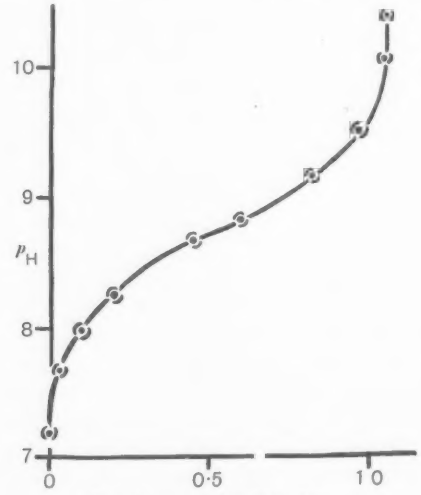
Titre	0	0·50	1·00	2·00	2·50
p_H	7·20	8·51	9·47	10·42	10·64

From Table V a correction curve was drawn and from it the values in the horizontal row headed "blank" in the table below were obtained.

Table VI. *Corrected titrations of* 2 % *fresh haemoglobin with* 0·360 N *NaOH.*

cc. NaOH	0	0·20	0·40	0·60	1·00	1·20	1·60	2·00	2·60	3·00
p_H	7·18	7·66	7·98	8·25	8·66	8·81	9·15	9·50	10·05	10·38
Blank	0	0·17	0·30	0·40	0·55	0·60	0·78	1·03	1·55	1·94
Corrected titre	0	0·03	0·10	0·20	0·45	0·60	0·82	0·97	1·05	1·06

From the corrected titration curve (Fig. 2) it will be seen that the 0·4 g. of haemoglobin present in 20 cc. of 2 % solution is neutralised by 1·06 cc. of 0·360 *N* NaOH. From this it is calculated that in the equivalent of haemoglobin (mass 16,660) there are 16 free acidic groups.



Titre in cc. of 0·360 *N* NaOH.

Fig. 2. Formaldehyde titration of 2 % fresh haemoglobin.

SUMMARY.

(1) It is found that in the equivalent of haemoglobin (taken to have mass 16,660) there are 24 or 25 free basic groups and 16 free acidic groups.

(2) Furthermore it is found that heat-denaturation is without effect on the number of free basic groups and without detectable effect on the number of free acidic groups of the haemoglobin molecule.

(3) From these results the conclusion is drawn that denaturation involves the hydrolysis of linkages of a character analogous to those involved in the hydrolysis of polysaccharides, as distinct from rupture of the polypeptide link (—CONH—). This conclusion is in agreement with earlier papers by the writer.

In conclusion, the writer desires to express his indebtedness to the Department of Scientific and Industrial Research for a grant which enabled the present research to be carried out. Further, the writer would like to acknowledge the benefit of communications with Dr L. J. Harris, and of the helpful advice of Prof. W. C. M. Lewis, under whom these investigations have been made.

REFERENCES.

Harris (1923, 1). *Proc. Roy. Soc. Lond.* B. **94**, 426.
—— (1923, 2). *J. Chem. Soc.* **123**, 3294.
—— (1924, 1, 2). *Proc. Roy. Soc. Lond.* B. **95**, 440, 500.
Kerridge (1925). *Biochem. J.* **19**, 611.
Lepeschkin (1922). *Kolloid Z.* **31**, 3342.
Lewis (1926). *Biochem. J.* **20**, 965, 978, 984.
Lindemann and Keeley (1924). *Phil. Mag.* **47**, 577.
Mathews (1924). Physiological Chemistry, 3rd ed., p. 110.
Robertson (1918). The physical Chemistry of Proteins.
Van Slyke and Birchard (1913). *J. Biol. Chem.* **16**, 539.
Sørensen and Jurgensen (1910). *Compt. Rend. Lab. Carlsberg*, **10**, 49.
Wu and Wu (1925). *J. Biol. Chem.* **44**, 369.

IX. THE FATE OF DESOXY-GLUCOSE IN THE RABBIT.

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(*Received November 23rd, 1926.*)

It has been shown [Winter, 1926] that the injection of glucal into rabbits during insulin convulsions brings about complete recovery to normal with raising of the blood-sugar. The possible lines on which glucal is effective in restoring the animal may be, (1) direct oxidation, (2) conversion into glucose or mannose, (3) formation of desoxy-glucose, which is a reducing sugar, but which differs from glucose in that two H atoms are attached to the second carbon. The possibility of the metabolism of desoxy-glucose has been tested by injection into rabbits in convulsions. The substance was prepared by the method of Bergmann, Schotte, and Lechinsky [1922], by the action of acid on glucal. The glucal was however allowed to crystallise, and was drained on a porous tile before treatment with acid. A few grams of the diphenylhydrazone [Bergmann and Schotte, 1921] were first prepared, and the compound decomposed with benzaldehyde. By this means a crystalline sample of the sugar was readily obtained, and this was used for nucleating the main preparation. The final product gave $[\alpha]_{5461} = +59{\cdot}5°$. The sugar was dissolved in a little water, and injected into the animals when the convulsions were well established. In one case a temporary improvement in the condition of the animal was noted, soon followed by a relapse. In no case has complete recovery been effected, though the subsequent administration of glucose will restore the animal permanently, showing that the desoxy-glucose has no toxic effect. There is a rise in the value for the blood-sugar following the injection of desoxy-glucose, but since there is little alteration in the condition of the animal it is probable that the increased amount of reducing substance is almost entirely due to the presence of the unaltered compound, and that the substance is not capable of metabolism by the tissues. The urine is found to give a green pine-shaving reaction, and in the HCl modification of the α-naphthol test a purple colour develops as soon as the mixture is warmed. It has been found that glucal and desoxy-glucose behave like the pentoses in respect to this reaction. There is however no possibility of confusion if pentoses are estimated in the presence of glucal, since the distillation of 0·03–0·05 g. of glucal with hydrochloric acid gave no weighable precipitate on adding phloroglucinol and allowing to stand. The colour reaction with

α-naphthol is evidently not due to the formation of furfural. The urine after shaking with charcoal reduced Fehling's solution vigorously. In one case a Wood-Ost estimation, which is less likely to be affected by nitrogenous compounds, indicated 0·33 % of sugar reckoned as glucose, following the administration of desoxy-glucose. Since the animal had not recovered from the insulin convulsions, it is likely that the main reduction was due to the injected sugar. The excretion of glucose during insulin hypoglycaemia is inconceivable. No precipitate of diphenylhydrazone could however be obtained when an alcoholic solution of diphenylhydrazone was added to the urine, the mixture being filtered and allowed to stand after shaking for 4 hours.

Weight kg.	Time	Protocols	Blood-sugar %
1·5	9.50	—	0·085
	10.00	3 mg. ins. HCl	—
	2.25	2 mg. ins. HCl	—
	3.20	—	0·059
	6.00	Convulsions	0·050
	6.20	0·5 g. desoxy-glucose	—
	7.45	Still collapsed. 0·5 g. desoxy-glucose	—
	9.15	Violent convulsions	0·060
	9.30	2 g. glucose	—
	10.30	Appeared normal	0·083
1·1	10.00	—	0·091
	10.10	3 mg. ins. HCl	—
	1.00	Convulsions	0·034
	1.20	1·0 g. desoxy-glucose	—
	2.00	Better, able to eat	—
	2.30	Collapsed	—
	2.45	1·0 g. desoxy-glucose	—
	4.45	—	0·11
	6.45	Still collapsed	0·062
	8.30	Animal died	—
1·1	9.45	—	0·089
	9.55	3 mg. ins. HCl	—
	12.30	Convulsions	0·041
	12.45	1·0 g. desoxy-glucose	—
	2.45	Collapsed	0·058
	4.00	Animal died	—

REFERENCES.

Bergmann and Schotte (1921). *Ber. deutsch. chem. Ges.* **54**, 440.
Bergmann, Schotte and Lechinsky (1922). *Ber. deutsch. chem. Ges.* **56**, 158.
Winter (1926). *Biochem. J.* **20**, 668.

X. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

II. THE SERIES OF RYVOSH.

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(*Received November 23rd, 1926.*)

The series of Ryvosh is concerned with the order in which the erythrocytes of the mammalia may be placed with respect to haemolysis by saponin and hypotonic saline respectively. Ryvosh [1907] places the types of cell in the following order, the most resistant species occurring first.

Saponin. Sheep, goat, ox, cat, grey mouse, pig, grey rat, dog, white rat, rabbit, guinea-pig.

Hypotonic saline. Guinea-pig, white rat, dog, grey rat, rabbit, pig, grey mouse, cat, ox, goat, sheep.

It will be observed that the order of resistance to saponin is the reverse of that to hypotonic saline, with the rabbit forming an exception.

This antagonism between the resistance to saponin and that to hypotonic saline has often been remarked upon, and various reasons have been put forward as to why it should exist. In particular, Orahovats [1926] has recently referred to the series in connection with his researches on the resistance of red cells in splenic blood, and as a result of these researches the subject assumes considerable importance. The results of Ryvosh having been obtained by methods which are open to considerable objection, we propose to investigate the matter afresh by a more satisfactory experimental procedure.

The objections to the original methods are principally the three following.

(1) Ryvosh added defibrinated blood to his solutions of NaCl and of saponin. The haemolytic systems accordingly contained serum, the presence of which renders the measurements of resistance to saponin unreliable, for serum itself inhibits saponin haemolysis [Ponder, 1923].

(2) The amount of blood thus added was in all cases such as to produce a 1 % suspension of cells. Now for experiments such as these, we can prepare suspensions in one of three ways. (*a*) We may have, in the case of the suspension from each animal, 1 cc. containing the cells from a constant volume of blood. This is the manner in which the suspensions used by Ryvosh were prepared. (*b*) We may arrange the suspensions so that each contains, in 1 cc., a constant number of cells. (*c*) We can make the suspensions so that 1 cc. of each contains a number of cells which present constant surface. To

decide which of these three alternative methods is the best is a difficult matter. The first is the most arbitrary, and in the case of saponin haemolysis the third probably the best, since the kinetics of saponin haemolysis are described by expressions which contain terms relating to the surface of the cells [Ponder, 1926, 1]. In this paper we shall, however, consider the resistances of suspensions prepared according to methods (*a*), (*b*) and (*c*), in preference to selecting one of these methods to the exclusion of the others.

In the case of haemolysis by hypotonic saline this difficulty does not arise, since the mechanism of the haemolysis is entirely different from that of saponin lysis, and since in the case of haemolysis by hypotonic saline we are not dealing with a process in which lysin is used up. We can accordingly compare, as Ryvosh does, suspensions containing the cells from a constant volume of blood.

(3) In the series of Ryvosh we have the members arranged merely according to order, and the order of resistance to saponin the reverse of that to hypotonic saline. When we compare the series for saponin with that for hypotonic saline and arrive at the conclusion that the one is the reverse of the other, we imply that if the members of the series are correlated according to rank, we shall find a coefficient of rank correlation of -1. It is well known, however, that the significance of such a value of a coefficient of rank correlation may be very much less than it appears to be, for the coefficient of rank correlation may differ very considerably from the coefficient of real correlation. We therefore seek to replace the arrangement by order by an arrangement according to some absolute measurement, so that we may be able to find the coefficient of real correlation, the value of which is the only reliable guide.

Methods.

Saponin haemolysis. The method used for the measurement of the relative resistance of various types of cell to saponin haemolysis has been fully described in previous papers [Ponder, 1926, 1, 2, 3]. In brief, it consists of plotting the time-dilution curves for the action of saponin on some type of cell selected as a standard—in these experiments, the cells of man—and on the type of cell whose relative resistance is required. The asymptotes of the two curves are found, and the concentrations of lysin corresponding to these asymptotes written down; the division of the one figure by the other supplies a resistance constant R, the magnitude of which gives the resistance of the one type of cell in terms of that of the standard type. The method is somewhat involved, but exceedingly accurate, and has the advantage that it expresses the resistance as the ratio of two absolute quantities. Moreover, R is constant not only for the concentrations corresponding to the asymptotes, but for all other concentrations of lysin, and thus the disadvantage of employing an arbitrarily selected concentration of lysin, or of estimating haemolysis after an arbitrarily selected time, is overcome.

All measurements are carried out at 25°; the saponin used is Merck's pure white saponin. The cell suspensions are prepared in the manner described in previous papers, and suitably diluted so as to contain either (*a*) the cells from a constant volume of the animal's blood, (*b*) a constant number of cells, or (*c*) a number of cells which present a constant surface. The dilution necessary is to be found from the figure for the red cell count per mm.^{3} of blood, and the figure for the surface of the cell as calculated from photographic measurements.

Hypotonic saline. In order to ascertain the resistance of different types of cell to hypotonic saline, a special series of solutions of varying tonicity is prepared. These are adjusted so as to give convenient tonicities when 0·2 cc. of suspension—consisting of cells suspended in 0·8 % NaCl—is added to 1·8 cc. of each solution. The following series is suitable.

cc. of 0·8 % NaCl	Resulting tonicity g. NaCl %	cc. of 0·8 % NaCl	Resulting tonicity g. NaCl %
0	0·08	44·4	0·40
5·5	0·12	50·0	0·44
11·1	0·16	55·5	0·48
17·5	0·20	61·1	0·52
22·2	0·24	66·6	0·56
27·5	0·28	72·0	0·60
33·3	0·32	77·7	0·64
39·0	0·36	83·3	0·68

To the quantity of 0·8 % NaCl, as shown in this table, there is added distilled water to 100 cc.; the result is a solution which gives, when to 1·8 cc. of it is added 0·2 cc. of the suspension in 0·8 % NaCl, a tonicity as shown by the corresponding figure in the table. The use of these solutions is much more convenient than is the drop method of Hamburger. If tonicities intermediate to those given in the table are required, they are easily obtained by mixing two of the solutions in the proper quantities.

The method used is to add to 1·8 cc. of each of the solutions 0·2 cc. of a suspension prepared by suspending the thrice washed cells from 1 cc. of blood in 20 cc. of 0·8 % NaCl, the entire experiment being carried out at 25°. After 60 minutes the tubes are examined, and the greatest tonicity which brings about complete haemolysis is noted. Solutions which give tonicities near this figure are now prepared, and with them the experiment is repeated, so as to determine the greatest tonicity which gives complete haemolysis to 0·01 % of NaCl.

Certain observations on this technique have to be made. (1) Brinkmann [1922] has pointed out that, for tonicity experiments in general, it is better to use solutions containing NaCl, KCl, $CaCl_2$, and $NaHCO_3$, than to use solutions of pure NaCl. The tonicity is varied by altering the NaCl content, the other components being kept the same. In this series of experiments we do not follow Brinkmann's suggestion for the following reason. It is well known that the most suitable balance of NaCl, KCl and $CaCl_2$ varies greatly for the erythrocytes of the different mammals; a solution such as Brinkmann recommends, although excellent for the study of the resistance of one particular

type of cell, will accordingly be unsuitable when cells of different species are to be compared, for the particular NaCl, KCl and $CaCl_2$ balance which is suitable for one animal may be very unsuitable for another, and thus we should obtain effects not directly connected with that of hypotonicity. We accordingly use pure NaCl. (2) We measure the resistance by the greatest tonicity which will complete haemolysis in 60 minutes in order to proceed in the same general way to that by which we measure the resistance to saponin. In the latter case we determine the asymptote of a time-dilution curve; here too, we determine what corresponds to an asymptote, for we imagine, although we do not actually do it, that we are plotting tonicity against the time taken to produce complete lysis, and we select that tonicity which produces complete lysis in 60 minutes—a time which, in these experiments, is as near infinity as we require. The justification of this step is that a tonicity a little greater than that selected would never complete haemolysis at all. (3) It is very important to remember that in these experiments, as in those determining the resistance to saponin, we are determining the resistance of the most resistant cells of the sample, and not of the cells of average resistance. The determination of the resistance of the average cell of the sample would be an exceedingly difficult matter. The importance of this point is that when we find a series in which the various cells may be placed with respect to their resistance, this series only holds for the most resistant cells; unless the standard deviation is the same for every suspension, the series may not hold for the average cells of the various suspensions.

Coefficients of correlation. These are calculated in the usual way. The coefficient of real correlation, r, is given by

$$\frac{S(xy)-Nd_1d_2}{\sqrt{\{S(x^2)-Nd_1^2\}}\sqrt{\{S(y^2)-Nd_2^2\}}},$$

where x and y are measured from points distant d_1 and d_2 from their respective means, and the coefficient of rank correlation, ρ, by

$$1-\frac{S(g^2)}{\frac{1}{6}N(N^2-1)},$$

where $S(g)$ denotes the sum of the gains in rank of the second series over the first.

Results.

1. *Suspensions containing cells from constant volume of blood.*

Each suspension was prepared so that the thrice washed cells from 1 cc. of the blood of the animal were finally suspended in 20 cc. of saline. The resistances to saponin and to hypotonic saline were determined by the above methods; in the table which shows the results the resistance to saponin is indicated by the figure for the asymptote of the time-dilution curve, the dilution of lysin being converted, for convenience in calculation, into the corresponding concentration of lysin in mg. The value of R is also given, the

cells of man being taken as the arbitrary standard. The resistance to hypotonic saline is given as the percentage of NaCl which gives the greatest tonicity capable of producing complete lysis in 60 minutes.

Table I.

	Resistances			Rank	
	Saponin				
Animal	Asymptote	R	Saline	Saponin	Saline
Man	0·030	1·0	0·35	3	1
Guinea-pig	0·033	1·1	0·40	4	2
Rat	0·021	0·7	0·40	2	3
Rabbit	0·012	0·4	0·42	1	4
Dog	0·036	1·2	0·44	5	5
Pig	0·039	1·3	0·45	6	6
Cat	0·063	2·1	0·48	7	7
Ox	0·198	6·6	0·49	9	8
Goat	0·075	2·5	0·52	8	9
Sheep	0·210	7·0	0·56	10	10

An inspection of this table will show that the different types of cell fall, as regards their resistance to hypotonic saline, in Ryvosh's series. As regards their resistance to saponin, they fall in a series which is nearly the reverse of the first, exceptions being provided by the goat, rabbit, and rat. Ryvosh gives the rabbit as the only animal which falls out of place in the series, for he finds the resistance of the cells of the goat greater than that of ox cells to saponin; in general, however, the order in which Ryvosh places the different types of cell is confirmed.

We now look at the correlation coefficients. Taking the series as given by Ryvosh, and correlating according to rank, we obtain a value of ρ of $-0{\cdot}94$. This value is very high, as might be expected from the fact that the order for resistance to saponin is the reverse of that for resistance to hypotonic saline, with the one exception of the rabbit. Taking next the series as found in Table I, and correlating according to rank, we get a value of ρ of 0·88, to which a negative sign must be prefixed, as the figure giving the resistance to saline becomes greater as the resistance becomes less. Next taking the correlation of the resistances as measured in absolute quantities, we obtain a much lower figure for r, the coefficient of real correlation, for it works out as 0·75, to which, again, a negative sign is to be attached.

2. *Suspensions containing a constant number of cells.*

In this series of experiments, each suspension was so prepared as to contain $2{\cdot}5 \times 10^8$ cells in 1 cc. In the table showing the results, the same arrangement as in Table I is adopted, and there is added a column to show the number of cells present in 1 mm.^{3} of the blood of each animal examined. This last figure was obtained by a red cell count made in the usual way.

Table II.

Animal	Cells per mm.^{3} $\times 10^{-6}$	Resistances: Saponin: Asymptote	Resistances: Saponin: R	Resistances: Saline	Rank: Saponin	Rank: Saline
Man	5·0	0·030	1·0	0·35	3	1
Guinea-pig	5·0	0·033	1·1	0·40	5	2
Rat	7·0	0·019	0·6	0·40	2	3
Rabbit	6·0	0·011	0·4	0·42	1	4
Dog	7·0	0·032	1·1	0·44	4	5
Pig	5·0	0·039	1·3	0·45	6	6
Cat	6·0	0·057	1·9	0·48	8	7
Ox	6·0	0·180	6·0	0·49	10	8
Goat	15·0	0·045	1·5	0·52	7	9
Sheep	10·0	0·140	4·6	0·56	9	10

The change in the method of preparing the suspension, it will be observed, causes a very considerable difference in the order of resistance to saponin. The coefficient of rank correlation now works out at $-0{\cdot}80$, and the coefficient of real correlation at no greater figure than $-0{\cdot}68$.

3. *Suspensions presenting constant area.*

In order to prepare these suspensions, one has to take account of the number of cells per mm.^{3} of blood, and the surface area of each cell. The first figure can be found by a count in the usual way; the second must be determined by calculation from the figures for the diameter and thickness of the cells. These can be obtained from photographic measurements of the erythrocytes suspended in plasma; the surface area is then calculated from the expression

$$\text{Area} = 2\pi A^2 + 2\pi AB\,\frac{\sinh^{-1} e}{e}.$$

A and B are the semi-axes major and minor of the cell, the biconcavities being imagined to be turned inside out, so that the cell assumes the form of a spheroid. The eccentricity about the minor axis is e, and it may be pointed out that the part of the expression, $\sinh^{-1} e/e$, is remarkably constant for all the cells of the mammalia, being equal to 0·6.

Multiplying the number of cells per mm.^{3} of blood by the figure for the area of each cell in μ^2 gives a figure which is proportional to the surface area presented by a suspension containing the cells from 1 cc. of the animal's blood. This figure may be compared with a similar figure for a suspension of human cells, which it is convenient to take as an arbitrary standard; in this way a series of suspensions from the blood of different animals can be prepared, each presenting the same surface to the lysin. In this series of experiments, the surface presented by the cells of 1 cc. of any of the suspensions used was $30 \times 10^9\ \mu^2$.

In Table III the value of a constant S, denoting the ratio of the surface presented by the cells in 1 cc. of the animal's blood to the surface presented by the cells in 1 cc. of human blood, is given in order that the strength of the various suspensions may be readily compared.

Table III.

		Resistances			Rank	
		Saponin				
Animal	S	Asymptote	R	Saline	Saponin	Saline
Man	1·0	0·030	1·0	0·35	3	1
Guinea-pig	0·8	0·039	1·3	0·40	5	2
Rat	1·0	0·021	0·7	0·40	2	3
Rabbit	0·95	0·012	0·4	0·42	1	4
Dog	1·0	0·036	1·2	0·44	4	5
Pig	0·53	0·050	1·7	0·45	6	6
Cat	0·62	0·085	2·8	0·48	7	7
Ox	0·66	0·230	7·7	0·49	9	8
Goat	0·72	0·090	3·0	0·52	8	9
Sheep	0·7	0·250	8·3	0·56	10	10

The order of resistance to saponin under the conditions of this experiment is very nearly the same as that shown in Table I, for there is a tendency for the greater number of cells, as found in the sheep and goat, to cancel out with a small figure for the surface area.

The coefficients of correlation work out as follows: $\rho = -0{\cdot}85$, $r = -0{\cdot}76$. These are very close to the values obtained from Table I, and considerably higher than those obtained from Table II.

Discussion.

Regarding the coefficients of rank correlation, we may set these aside at once. The high value obtained from Ryvosh's original series, and the not much lower value obtained from Table I, are quite misleading, and no deductions can be drawn from them except that the order in which the various types of cell fall with respect to resistance to saponin and to hypotonic saline is extremely unlikely to be brought about by mere chance. The odds against this order occurring by chance alone, are, in fact, about 6000 to 1.

We are also inclined to set aside the results of Table II, with a value of r of $-0{\cdot}68$. There appears no good reason for comparing suspensions containing a constant number of cells, when we have a comparison between suspensions presenting constant surface to the lysin. In any case, the value of r obtained by this method of comparison is the lowest of all; if the comparison of suspensions containing a constant number of cells should be insisted upon, the remarks about to follow would apply *a fortiori*.

Tables I and II each yield a value of r of about $-0{\cdot}75$. This is a high value for a coefficient of correlation, but not so high as to justify us in saying that there is only one factor determining the resistance—a factor which gives a high resistance to saponin and a low one to hypotonic saline. It appears permissible, however, to say that one important factor must exist, coupled with perhaps one, or more, subsidiary factors, the operation of which may affect, in one direction or the other, the operation of the primary factor.

The nature of this primary factor is a matter for interesting speculation. Port [1922] has suggested that the phosphoric acid content of the cell is the

essential factor in determining the resistance, for he observes that the order of resistance to saponin for the cells of different animals is the same as the order of phosphoric acid content as given by Abderhalden [1895], while the order of resistance to hypotonic saline is the reverse of the order of phosphoric acid content. In applying this suggestion to his own researches Orahovats apparently takes the term "phosphoric acid" to include inorganic phosphates; with this interpretation, he finds little in support of Port's suggestion.

Examining Abderhalden's table, we find four sets of figures which bear on this point; figures are given for inorganic phosphoric acid, total phosphoric acid, phosphoric acid as nuclein, and lecithin. Although only seven animals appear in common to both Abderhalden's table and Table I of this paper, a close examination of these figures throws considerable light on the suggestion of Port.

First we correlate, according to rank, the total phosphoric acid content and the resistance to saponin. This gives a coefficient of $-0{\cdot}84$, which is very high. This total phosphoric acid, it is now to be observed, may be broken up into the inorganic and the organic phosphoric acid, for both of which figures may be obtained from Abderhalden's table. Further, the principal sources of the organic phosphoric acid are nuclein and lecithin, for both of which Abderhalden gives figures. Correlating according to rank with the resistance to saponin, we find the following coefficients:

Total phosphoric acid and resistance	$-0{\cdot}83$
Inorganic phosphoric acid and resistance	$-0{\cdot}52$
Organic phosphoric acid and resistance	$-0{\cdot}53$
Lecithin and resistance	$-0{\cdot}21$
Phosphoric acid as nuclein and resistance	$-0{\cdot}89$

These being merely rank correlations, it is difficult to attach a significance to the figures, but one thing stands out clearly—the correlations are all negative, the greater content of any one of these substances being associated with the lower resistance to saponin.

This fact is very important, for it has been established with a considerable degree of certainty that saponin enters into a combination with some component of the cell, from which we should expect to find the amount of that component increasing with the amount of saponin required to be used up to bring about lysis, and the amount of that component increasing with the resistance. This would give, of necessity, a positive correlation, whether by rank or by absolute value, and not a negative one.

We have already suggested [Ponder, 1926, 1] that the component with which the saponin interacts is a protein component of the cell wall, and so we may correlate the resistance to saponin with the protein contents of the various cells, as given by Abderhalden, excluding, of course, the content of haemoglobin. This procedure at once gives the high positive coefficient of 0·79. The positive sign, moreover, gives the coefficient a real significance, for we can say that the more protein in the cell, the more saponin requires to be

used up to bring about lysis, and the more resistant is the type of cell accordingly. Or, reversing the argument, the more protein in the cell, the more readily is the cell haemolysed with hypotonic saline.

We suggest that it is the protein content which is the primary factor in the determination of the resistance to saponin and to saline, and that the observation of Port is due to the fact that there exists a coefficient of rank correlation between protein content and phosphoric acid of $-0{\cdot}75$. Why this latter figure should appear we do not know, nor is its appearance material to the question at issue. The suggestion that the protein content is the essential factor at least brings Ryvosh's series into line with the results of previous researches, all of which indicate that the action of saponin is on the protein component of the cell. The phosphoric acid content happens to be a guide to the resistance merely because the amount of this substance varies inversely, roughly speaking, with the protein content.

To express the conclusion concisely, the evidence points to the following state of affairs. In a cell such as that of the sheep, rich in protein, much saponin requires to be transformed by the formation of a compound with this protein before lysis is brought about. The cell is therefore very resistant to saponin. The same cell is readily haemolysed by hypotonic saline, whether because of its high protein content, or for some other reason which we cannot indicate. A cell poor in protein, such as that of the rat, requires little saponin for the transformation of sufficient of the cell wall to bring about haemolysis, and so appears relatively unresistant to saponin. At the same time, it requires a relatively low tonicity to bring about lysis, and the cell is thus relatively resistant to hypotonic saline.

There is no difficulty in appreciating why a large protein content should give a high resistance to saponin. The reason why a high protein content should accompany a low resistance to hypotonic saline is more difficult to discover, and must be sought in the mode of operation of hypotonic saline on the cell. It may be that, just as the resistance to saponin is determined by the protein content but indicated by the phosphoric acid content, so the resistance to hypotonic saline is indicated by the protein content, but determined by some factor which is negatively correlated to the amount of protein in the cell.

SUMMARY.

1. The series of Ryvosh is investigated afresh by quantitative methods. Ryvosh's results are in the main confirmed, and his conclusions amplified by the calculation of the correlation coefficients applicable to the series.

2. It is suggested that the resistance of cells to saponin is principally determined by the protein content of the cell, exclusive of haemoglobin, and the evidence for this suggestion is discussed.

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REFERENCES.

Abderhalden (1895). *Z. physiol. Chem.* **25**, 67.
Brinkmann (1922). *Ned. T. v. Gen.* **66**, 2464.
Orahovats (1926). *J. Physiol.* **61**, 447.
Ponder (1923). *Proc. Roy. Soc. Lond.* B, **95**, 42.
—— (1926, 1). *Proc. Roy. Soc. Lond.* B, **100**, 199.
—— (1926, 2). *Proc. Roy. Soc. Lond.* B, **99**, 461.
—— (1926, 3). *Biochem. J.* **20**, 507.
Port (1922). Höber's Physikalische Chemie, p. 593.
Ryvosh (1907). *Pflüger's Arch.* **116**, 229.

XI. THE BIOCHEMISTRY OF THE AQUEOUS HUMOUR.

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The view at present most generally accepted regarding the origin and nature of the aqueous humour of the eye is that it is secreted by the cells of the ciliary epithelium, that it circulates through the eye, and that it finds an exit therefrom largely by way of the canal of Schlemm. Alternatively it is held by some that this fluid is formed from the blood by a process of simple transudation through the capillary walls and circulates in a similar manner. The evidence whereon the secretory theory is based is largely anatomical, resting on the histological structure of the ciliary epithelium and its resemblance to a gland—a form of evidence at best inconclusive and susceptible of varying interpretations. Some animals possess no ciliary body at all; others, possessing a ciliary body, show no specific glandular formation; and finally, in those animals (*e.g.* man) wherein the anatomical evidence is most complete, the resemblance to true glands is questioned by many authorities. Moreover, even in these latter it can be shown that the formation of the intra-ocular fluids, though largely associated with the ciliary body, is by no means confined to it. The "circulation" of the aqueous humour has been determined and measured only by unsound physiological experiments wherein the normal pressure conditions have been upset by opening the eye, or by making injections into the eye, or by employing other procedures which can be interpreted as calculated to initiate artificially an abnormal circulation.

In a series of experiments on the intra-ocular pressure it was indicated [1926, 1] that the pressure equilibrium of the eye might be explained more consistently on the hypothesis that it was of a hydrostatic rather than of a hydrodynamic nature. These suggested the probability of a third hypothesis which has more recently been put forward, that the aqueous humour is formed by dialysis from the blood, and that, apart from a minimal circulation determined by metabolic interchange and muscular activity, it is in equilibrium with it. A further series of experiments on the vascular pressures of the eye [1926, 3] demonstrated that the physical forces involved in the production of the aqueous humour did not necessitate the intervention of any secretory energy; the difference between the intra-ocular pressure and the hydrostatic pressure in the ocular arteries and veins, and the difference in

osmotic pressure between the aqueous humour and the capillary plasma were found to be compatible with a dialysis hypothesis. It was shown also that the pressure equilibrium forbade a hydrostatic outflow of aqueous humour into the veins under normal pressure conditions [1926, 2], and that, in addition, the differences in osmotic pressure [1927] and electrical potential between the plasma and the aqueous humour were of an order such as to satisfy the requirements of a system in thermodynamical equilibrium.

In complicated physiological experiments the number of imperfectly controllable variables frequently leads to equivocal results and rarely to a pragmatic conclusion; whilst the attempt to eliminate the variables tends to introduce conditions so abnormal as to defeat its own ends. It was therefore felt that the study of the aqueous humour from the biochemical point of view would not be without interest. The main difficulty which presented itself was the small quantity of intra-ocular fluid which is available from any one animal, and the minute concentration of some of the constituents. In order to get a general comparison between the aqueous humour and the blood, large quantities of horse aqueous humour were obtained, and the pooled fluid was analysed concurrently with a typical sample of horse serum. By this procedure a qualitative comparison could be made, and though it cannot lay claim to quantitative accuracy, a relatively close approximation can be obtained. The principal constituents of each class of substance were then selected and micro-estimations made of these, comparing each with the corresponding concentration in the arterial and venous plasma of the same animal. Finally, changes in the chemical constitution of the aqueous humour were studied under experimentally produced variations in the blood and in the eye.

I. The general chemistry of the aqueous humour.

A considerable amount of work has been done on the general chemistry of the aqueous humour—in the horse notably by Mestrezat and Magitot [1921]—but in no case has a systematic analysis been directly compared with the blood.

Collection of material. The aqueous humour was taken from the eyes of horses immediately after their being slaughtered. In this animal each eye contains 1 to 2 cc. of fluid; only the first cc. was withdrawn, since on complete evacuation the last drawn fluid becomes contaminated with plasma exuded from the capillaries. The cornea was cleaned and dried, a nick made through three-quarters of its substance with a sharp knife, and the needle of a 1 cc. syringe inserted through it, care being taken to avoid the iris and to cause as little pressure disturbance as possible. The serum was a typical sample taken from the farm of the Medical Research Council.

The chemical analysis is given in the accompanying table.

Methods of analysis. The total protein was estimated in the aqueous humour by acidifying 100 cc. and coagulating by heat; the precipitate was washed with water and alcohol, dried at 100° and weighed. 10 cc. of serum

Quantities in g. per 100 cc.

	Aqueous humour	Serum
Water	99·6921	93·3238
Solids (dried at 100°)	1·0869	9·5362
Total protein	0·0201	7·3692
Albumin	0·0078	2·9557
Globulin	0·0123	4·4135
Non-protein N	—	0·0239
Total N	0·0268	—
Urea	0·028	0·027
Amino-acids	0·029	0·035
Creatinine	0·002	0·002
"Fats"	Trace (0·004)	0·13
Cholesterol	? Nil	—
"Sugar"	0·0983	0·0910
Sodium	0·2787	0·3351
Potassium	0·0189	0·0201
Calcium	0·0062	0·0101
Magnesium	0·0026	0·0028
Chlorine	0·4371	0·3664
Inorg. P (P_2O_5)	0·0033	0·0030
Inorg. S (SO_4)	0·0061	0·0058

diluted to 100 cc. with distilled water were treated similarly. The albumin was determined by precipitating the globulins by adding an equal volume of saturated ammonium sulphate solution to 100 cc. which had been allowed to concentrate in a desiccator, allowing this to stand for 24 hours, and filtering; the filtrate was acidified with $N/10$ sulphuric acid and filtered; the precipitate was redissolved in the original volume of distilled water, neutralised with dilute caustic soda, and re-precipitated with half-saturated ammonium sulphate, the albumin being precipitated again from the filtrate with acid. This process of fractionation was repeated five times. The final filtrate was acidified and boiled, the precipitate washed with water, alcohol, and ether, dried at 100° and weighed. 10 cc. of diluted serum were treated similarly. The globulin was taken as the difference between the total protein and the albumin.

The total nitrogen of the aqueous humour was estimated by a micro-Kjeldahl determination; the non-protein nitrogen of the serum by the same method on a protein-free filtrate (Folin and Wu). Urea was estimated by the urease method: the amino-acids by Van Slyke's method: and the creatinine colorimetrically against a standard creatinine picrate solution.

By the term "fats" is meant the figures obtained by weighing an ether extract of dried residue. The cholesterol was estimated after extraction by precipitation with digitonin.

"Sugar" is taken as reducing substance estimated as glucose by the Schaffer-Hartmann method.

The total mineral ash was obtained by calcination. The material thus left was dissolved in HCl; barium chloride and baryta were added, and the phosphates and sulphates removed; barium salts were removed by ammonia and ammonium carbonate; the filtrate was evaporated to dryness and the ammonium salts removed by heat; the residue was dissolved, treated again with ammonia and ammonium carbonate, filtered, acidified with HCl and

evaporated to dryness. This residue was redissolved; the potassium was estimated by precipitation with H_2PtCl_6, and the amount of sodium calculated by difference. The calcium was precipitated as oxalate, and estimated as CaO, the magnesium as ammonium magnesium phosphate and estimated as magnesium pyrophosphate. The chlorides were determined by Ruszynák's modification [1921] of Koranyi's method; the inorganic phosphates gravimetrically as pyrophosphate; and the inorganic sulphates by conversion to benzidine sulphate and titration with sodium hydroxide.

The results of the chemical analysis show that the constituents of the aqueous humour, when compared with those of serum, may be divided into three groups of substances depending on the physical state of their molecules in solution.

1. *Colloidal substances. Partition coefficient serum/aqueous humour* > 1.

All the colloidal substances are found in the aqueous humour in much smaller concentration than in the serum. Proteins are present in the former in very small quantities; but it is to be noted that although this is the case the different fractions (as determined by precipitation by ammonium sulphate) are found in approximately the same proportion as they occur in the blood. Thus the albumin/globulin ratio in the former is 38·8/61·2, in the latter 39·9/60·1. Further it was shown by dialysing the globulin fraction against distilled water that in the aqueous humour as in the serum it was composed partly of euglobulin and partly of pseudoglobulin, although, unfortunately, the minuteness of the quantities involved precluded any reliable quantitative estimation. Moreover, it can be shown that they are specifically identical[1]. Similarly the "fats," also indiffusible substances, are found in correspondingly minute quantities in the aqueous humour.

Since all the colloidal constituents of the serum appear only in traces in the aqueous humour the two solutions are of very dissimilar molecular aggregation; in comparing the distribution of the diffusible constituents it is therefore necessary to apply a correction factor to allow for the difference in solid displacement due to the unequal mass of solute. Thus 100 cc. of the horse aqueous humour contain 1·0869 g. of solids and 99·6921 g. of water, whilst the same quantity of serum contains 9·5362 g. of solids (largely protein) and 93·3238 g. of water. A correction factor therefore of 100/99·6921 or 1·003 applied to the aqueous humour and of 100/93·3238 or 1·07 applied to the serum gives comparative results expressed as concentrations dissolved in an equal quantity (100 g.) of water.

2. *Diffusible non-dissociated substances. Partition coefficient* $= 1$.

The total N of the aqueous humour was found to be 0·0268 g. %. Deducting 0·0032 g. % to allow for 0·02 % protein, the non-protein nitrogen becomes 0·0236 g. %—a close approximation to the non-protein nitrogen

[1] Unpublished researches, at present being extended, with Dr Percival Hartley.

content of the serum (0·0239 g. %). The amino-acid content at any one time is probably too variable a quantity to permit reliable deductions to be made from it when the method of collecting materials is borne in mind. The creatinine appeared to be present in both in equal quantities. The urea and the "sugar" also appeared in practically the same concentrations.

	Aqueous humour		Serum	
	G. per 100 cc. solution	G. per 100 g. water	G. per 100 cc. solution	G. per 100 g. water
Urea	0·028	0·028	0·027	0·0289
"Sugar"	0·0983	0·0986	0·0910	0·0974

The diffusible non-dissociated substances are therefore partitioned between the two fluids in approximately equal amounts.

3. *Dissociated diffusible substances.*

The dissociated substances are seen to be unequally distributed, even when corrected for solid displacement. In each case the cations have a partition coefficient greater than one, and the anions a partition coefficient less than one.

	Aqueous humour			Serum		
	Per 100 cc. solution		Per 100 g. water	Per 100 cc. solution		Per 100 g. water
	g.	Milli-mols per litre	g.	g.	Milli-mols per litre	g.
Cations:						
Sodium	0·2787	121·2	0·2795	0·3351	145·6	0·3585
Potassium	0·0189	4·8	0·0190	0·0201	5·1	0·0215
Calcium	0·0062	1·5	0·0063	0·0101	2·5	0·0108
Magnesium	0·0026	1·1	0·0026	0·0928	1·2	0·0030
Anions:						
Cl'	0·4371	123·1	0·4384	0·3664	103·2	0·3920
PO_4'''	0·0044	1·38	0·0044	0·0040	1·26	0·0043
SO_4''	0·0061	1·8	0·0062	0·0058	1·7	0·0062

It is seen that in the serum there is a considerable excess of basic radicles, which is shown especially in the case of sodium and calcium. This is accounted for by the fact that in blood part of these are associated with protein as protein salts, and as such are rendered indiffusible. Rona and György [1913] found that 15 to 28 % of the total quantity of sodium was indiffusible for this reason, and Rona and Takahashi [1913] that 30 to 40 % of the total calcium was similarly in association with protein. Conversely, the acidic radicles show a predominance in the aqueous humour, the chlorides showing a higher relative concentration than the phosphates and sulphates. This again suggests comparison with the work of previous observers, since all the chlorides of serum have been found to dialyse [Ascher and Rosenfeld, 1907; Creveld, 1921], while the phosphates have been held back to some extent in loose combination with the protein. The increase in the concentration of anions as a whole and the diminution of cations strongly suggest that the distribution of the ionic activities between the aqueous humour and the blood is subject to the

thermodynamical laws relating to two fluids in the membrane equilibrium described by Donnan. Thus the relative concentrations expressed as normal chlorine and sodium are:

$$Cl_{aq} : Cl_{serum} = 123 : 103$$
$$Na_{aq} : Na_{serum} = 121 : 145.$$

The theoretical relation characteristic of such an equilibrium

$$[Na^+]_{aq} \times [Cl^-]_{aq} = [Na^+]_{serum} \times [Cl^-]_{serum}$$

therefore becomes $121 \times 123 = 145 \times 103$

or $148{\cdot}83 = 149{\cdot}35.$

The distribution of all the constituents of the aqueous humour thus appears to be determined by physical laws and provides no evidence of the expenditure of any secretory energy in its elaboration, whilst the concentration of its ionised substances seems to preclude a simple transudation, but rather suggests that it is formed by dialysis from the blood through a membrane which is almost impermeable to colloidal micelles and is in equilibrium with it.

II. The "sugar" and salt of the aqueous humour.

In order to compare the aqueous humour with the blood under more exact conditions than were possible in the method of collection of material detailed above, two of the most abundant and easily manipulated constituents were chosen—"sugar" as being representative of the non-dissociated substances, and chlorides as being representative of the ionised constituents—and their relative concentrations compared. The two fluids—aqueous humour and plasma—were in each case derived from the same animal and were retained in a condition approximating the normal as closely as possible.

Rabbits were used. The aqueous humour was withdrawn under sterile conditions by means of a syringe dried with alcohol and ether. The needle had a broad lance point which was introduced into the cornea obliquely near the limbus; such a needle is inserted with less disturbance than the ordinary round pointed instrument, and with the latter it was found difficult to prevent aqueous humour escaping round it at the moment of introduction. Cocaine (2 %) was instilled into the conjunctival sac as an anaesthetic; it has been repeatedly demonstrated that this procedure does not appreciably alter the properties of the intra-ocular fluids. Before the needle was introduced the cornea was dried with blotting paper to obviate any contamination with lacrymal secretion which is of widely different composition. Blood was taken also with aseptic precautions from the ear, the central artery or the marginal vein being used as the case required. Plasma in preference to serum was employed, since it is the former which comes into equilibrium with the aqueous humour *in vivo*; the use of anti-coagulants was dispensed with, since these substances are known to alter the distribution of its constituents to such an extent as to make estimations carried out *in vitro* useless for comparative

purposes with the actual state of the blood *in vivo*. Blood was therefore sucked directly through a paraffined needle and tube into a paraffined centrifuge tube under a layer of paraffin, and, after centrifuging, the middle layer of plasma was pipetted off without its ever having been in contact with air. The centrifuging was done rapidly, and in this way both arterial and venous plasma were obtained in a state as near to that in which they occur naturally as experimental manipulations permitted.

The results are expressed in g. per 100 cc. solution, and g. per 100 g. water. 100 cc. rabbit's plasma were found to contain 8·6832 g. solids (dried at 100°), and the density was 1·023. Correspondingly, 100 cc. aqueous humour contained 1·0899 g. solids (dried at 100°), and its density was 1·007. The factors 1·07 and 1·003 respectively therefore give the concentration of solution in 100 g. water.

The "sugar" (*i.e.* reducing substance) was estimated by the Hagedorn-Jensen method.

	No. of rabbit	G. per 100 cc. solution	G. per 100 g. water
Aqueous humour	1	0·141	0·141
	2	0·138	0·139
	3	0·175	0·175
Arterial plasma	1	0·136	0·145
	2	0·139	0·148
	3	0·170	0·182
Venous plasma	1	0·120	0·128
	2	0·111	0·118
	3	0·143	0·153
Average aqueous humour		0·151	0·151
„ arterial plasma		0·148	0·158
„ venous plasma		0·125	0·133

The sugar concentration of the aqueous humour lies between that of arterial and venous plasma, a relation which suggests that it comes into equilibrium with capillary plasma. The concentration appears to be more closely related to the arterial than to the venous plasma; this finding may be compared with that of Foster [1923], who showed that the sugar content of "finger blood," and therefore presumably of capillary blood, was very nearly identical with that of arterial and widely different from that of venous blood. The results obtained may be compared with those of previous observers on the sugar content of aqueous humour. Osborne [1919] found the concentration in both to be the same; Ask [1914] found the sugar in the aqueous humour to be 0·01 to 0·02 % greater than in the blood, Deiter [1925] 0·004 % greater; whilst Haan and Creveld [1921, 1, 2] obtained a value for the aqueous humour 0·045 % less than that for the blood. These last authors accept the hypothesis of the dialysis of the aqueous humour, and, taking the sugar content of the capillary plasma as being the mean of the arterial and venous plasma and finding the concentration in the aqueous humour less than this, they conclude therefrom that glucose is partially retained in combination with plasma proteins. Until more exact knowledge of the sugar content of capillary

blood is available it would seem dangerous to draw any such conclusions from the above figures. The results obtained in the present investigation suggest, however, that the sugar content of the aqueous humour lies between that of the arterial and venous plasma and support the hypothesis of the dialysis of the aqueous humour.

Salt. The chloride was estimated by Ruszynák's modification of Koranyi's method, and is expressed as NaCl.

	No. of rabbit	G. per 100 cc. solution	G. per 100 g. water
Aqueous humour	1	0·668	0·670
	2	0·597	0·599
	3	0·641	0·643
Arterial plasma	1	0·603	0·645
	2	0·543	0·581
	3	0·590	0·631
Venous plasma	1	0·578	0·618
	2	0·501	0·536
	3	0·573	0·613
Average aqueous humour		0·635	0·637
„ arterial plasma		0·579	0·619
„ venous plasma		0·551	0·589

The chloride content of the aqueous humour is higher than that of the plasma even when expressed as g. in an equal weight of water. The results obtained thus agree with those of Ascher [1922] and Creveld [1921]—and again suggest that the aqueous humour is a dialysate from the blood.

III. The chemical composition of abnormal aqueous humour.

If the intra-ocular fluids are a dialysate of the plasma there are two possible methods of changing their composition: by altering the permeability of the dialysing membrane (*i.e.* the capillary wall), and by altering the composition of the blood.

1. *Alteration of the permeability of the capillary walls.*

In a state of dilatation the permeability of the capillary walls is increased, and under these circumstances a larger proportion of colloidal molecules is able to penetrate through their walls. This occurs in the eye as elsewhere, and, along with the increased protein content of the aqueous humour, definite changes in the distribution of ionised substances occur.

(*a*) *Dilatation of the capillaries by paracentesis.* When the eye is punctured and the intra-ocular fluids are withdrawn the capillaries, deprived of the supporting pressure of the aqueous humour, undergo immediate dilatation. The fluid re-formed under these conditions I have elsewhere [1927] called "plasmoid aqueous." The aqueous humour was withdrawn from the eyes of rabbits, using the same technique as was employed previously, and the re-formed fluid was similarly withdrawn 20 minutes after the first paracentesis.

The chemical constitution of the normal and plasmoid aqueous humours was compared in respect of their colloid, sugar, and chloride contents.

Colloid content. The colloids, which are largely composed of protein, were estimated refractometrically. A dipping refractometer (Zeiss) was used provided with an auxiliary prism to enable it to deal with one drop of fluid and the temperature was kept constant by a thermostat; the error of the instrument is ± 3·7 units of the fifth decimal place of n_D.

The increase of refractivity found in the "plasmoid aqueous" shows an increase in its content of colloids (see table below)—a fact which has long been known. Considering the total colloid as proteins, the approximate corresponding percentages of this substance are also given. The figures do not pretend to any great accuracy. They were calculated by the technique suggested by Robertson [1915] applied to a large quantity (100 cc.) of horse aqueous humour. The refractive index of a protein-free preparation of this fluid was subtracted from that of the normal horse aqueous humour, and the differential result thus obtained was correlated with a gravimetric estimation of the total protein in the original fluid. That this refractive index was due to increase of protein, both albumin and globulin, was seen by special tests for these substances. The Noguchi globulin test gave a granular flocculent precipitate whilst Pandy's reaction gave a typical blue-white cloud; both these reactions are absent from the normal aqueous humour. Similarly the Nonne-Apelt test gave a positive reaction for globulin, and on filtering and acidifying the filtrate the presence of increased albumin was verified.

	Refractive index
Normal horse aqueous humour	1·335130
Protein-free horse aqueous humour	1·335091
Refractive index of protein	0·000039
Protein	0·024 %

The aqueous humour of the rabbit was found to have on the average a higher refractive index than that of the horse.

	Refractive index
Normal rabbit aqueous humour	1·335168
Protein-free rabbit aqueous humour	1·335091
Refractive index of protein	0·000077
Protein	0·04 % (approx.)

No. of rabbit	Aqueous removed cc.	n_D Normal aqueous	n_D "Plasmoid aqueous"	Difference (plasmoid – normal)	Approx. % protein
1	0·1	1·335244	1·337088	0·001844	1·0
2	0·2	1·335130	1·338428	0·003298	1·8
3	0·25	1·335168	1·339036	0·003868	2·0
4	0·32	1·335244	1·339834	0·004590	2·5

Concurrent sugar and salt estimations showed the following results. The sugar was estimated by the Hagedorn-Jensen method and is expressed as g. per 100 cc.

No. of rabbit	Normal aqueous	"Plasmoid aqueous"	Difference
1	0·143	0·148	0·005
2	0·155	0·173	0·018
3	0·173	0·175	0·002
4	0·165	0·172	0·007

The increase of sugar in the "plasmoid aqueous" is probably largely to be explained by an increase in the blood sugar occurring during the experiment due to emotional excitement on the part of the animals. An effort was made to accustom the rabbits to experimental procedures before performing any of the above experiments upon them; but without success. Thus in one experiment the blood sugar at the start was 0·154 %, and at the end had risen to 0·177 %.

The salt was estimated by the Ruszynák method and is expressed as g. NaCl per 100 cc.

No. of rabbit	Normal aqueous	"Plasmoid aqueous"	Difference
1	0·641	0·600	0·041
2	0·597	0·561	0·036
3	0·680	0·536	0·144
4	0·500	0·421	0·079

There is therefore a constant decrease in the quantity of chloride with increasing concentration of protein.

(*b*) *Dilatation of the capillaries by radiant energy.*

(i) *Ultra-violet rays.* Rabbit—10 minutes' irradiation at 2 feet from a quartz mercury vapour lamp, water-cooled. The left eye protected.

Rabbit	Left eye normal	Right eye irradiated
Refractive index	1·335168	1·340898 (approx. 3 % protein)
"Sugar"	0·154	0·172
Chloride	0·693	0·520

(ii) *Infra-red rays.* Rabbit—10 minutes' irradiation at 2 feet from a carbon arc, ultra-violet filtered off through 1 cm. thickness of glass. The left eye protected.

Rabbit	Left eye normal	Right eye irradiated
Refractive index	1·335247	1·341990 (approx. 3·5 % protein)
"Sugar"	0·125	0·131
Chloride	0·661	0·501

It is probable that both of these agencies act in the same way, the radiant energy being absorbed by the pigment of the iris, and there converted into heat which causes capillary dilatation.

(*c*) To show that the changed chemical constitution was due to the dilatation of the capillary walls, a paracentesis was done after 1 cc. of 1 : 1000 adrenaline with cocaine had been injected behind the eyeball. This counteracts to a large extent the dilatation caused by the paracentesis. Wessely [1908] first showed that the aqueous humour formed secondarily under these conditions contained less protein than the usual "plasmoid aqueous": the present analysis demonstrates that throughout it is to a less extent of a plasmoid nature, but resembles more closely the normal fluid.

Rabbit	Left eye—normal	Right eye—paracentesis after adrenaline
Refractive index	1·335130	1·335244
"Sugar"	0·152	0·175
Chloride	0·638	0·630

It is suggested that whilst the normal aqueous humour has the chemical constitution which one would expect were it in membrane equilibrium with the capillary plasma, the abnormal humour formed under experimentally varied conditions changes its composition in a corresponding manner. On increasing the permeability of the separating membrane and allowing more colloid to enter the eye, the increase is proportional to the amount of capillary dilatation produced. With the tendency to equalisation of the amount of colloid on either side of the membrane the excess of cations largely disappears, while, allowing for the increased glucose content of the blood which accompanies the excitement of the experimental manipulations, the non-dissociated constituents ("sugar") remain practically constant. Moreover, on preventing capillary dilatation the aqueous humour formed secondarily after puncture of the eye is not far removed from the normal. It would appear, therefore, that the view is unjustified which ascribes to the normal humour and the "plasmoid aqueous" fundamentally different origins—that the former is a secretion and the latter a transudation; it would seem rather that the intra-ocular fluids are formed under both conditions by the same process of dialysation through a membrane of varying permeability.

2. *By varying the chemical constitution of the blood.*

A series of experiments was done on cats wherein varying quantities up to 50 cc. of a 10 % solution of gum arabic were injected intravenously: no change in the refractive index of the aqueous humour was ever detected. Where colloidal substances are susceptible to delicate biological tests, however, their presence can be detected in proportions corresponding to the traces of protein normally present, as is seen in the case of the various substances associated with immunity [Poleff, 1914, and others]. Difficultly diffusible drugs are also found in traces—*e.g.* the organic compounds of arsenic [Neame and Webster, 1923]. All these substances are found in the "plasmoid aqueous" in quantity.

Diffusible substances find their way freely into the aqueous humour. Thus in experiments carried out on the cat [1926, 1] when 10 cc. of a 30 % glucose solution were injected intravenously, the sugar content of the right eye before the experiment was found to be 0·146 %, whilst that in the left 15 minutes after the injection was 0·204 %. Similarly with sodium chloride; the concentration of this substance was found to rise from 0·641 to 0·698 % after the intravenous injection of 10 cc. of a 30 % solution. This corresponds with the clinical findings of Ask [1914] and Deiter [1925], who found that the sugar content of the aqueous humour rose parallel with the blood in diabetics, and of Gala [1924], who found that in cases of chloride retention the aqueous humour varied similarly, and the experimental findings of Löhlein [1910] and Haan and Creveld [1921, 2], who found that such substances as fluorescein, potassium ferrocyanide, and potassium iodide were partitioned between the aqueous humour and the blood in the ratio of their diffusion constants.

SUMMARY.

From the biochemical point of view neither the normal nor the abnormal aqueous humour shows evidence of the existence of any special secretory mechanism in its elaboration but appears to be rather a dialysate of the capillary blood.

I am indebted to the Research Committee of St George's Hospital Medical School for financial assistance in the Sir Francis Laking Prize; and to Mr J. A. Gardner for advice and criticism.

REFERENCES.

Ascher (1922). *Arch. Ophthal.* **167**, 247.
Ascher and Rosenfeld (1907). *Biochem. Z.* **3**, 335.
Ask (1914). *Biochem. Z.* **59**, 1.
Creveld (1921). *Biochem. Z.* **123**, 304.
Deiter (1925). *Arch. Augenheilk.* **96**, 179.
Duke-Elder (1926, 1). *Brit. J. Ophthal.* **10**, 1.
—— (1926, 2). *J. Physiol.* **61**, 409.
—— (1926, 3). *J. Physiol.* **62**, 1; *Brit. J. Ophthal.* **10**, 513.
—— (1927). *J. Physiol.* In the Press.
Foster (1923). *J. Biol. Chem.* **55**, 291.
Gala (1924). *Bratis, Lek. Listy.* **5**, 42.
Haan and Creveld (1921, 1). *Biochem. Z.* **123**, 190.
—— —— (1921, 2). *Biochem. Z.* **124**, 172.
Löhlein (1910). *Arch. Augenheilk.* **65**, 318, 417.
Mestrezat and Magitot (1921). *Compt. Rend. Soc. Biol.* **84**, 185.
Neame and Webster (1923). *Trans. Ophthal. Soc., U.K.* **43**, 381.
Osborne (1919). *J. Physiol.* **52**, 347.
Poleff (1914). *Klin. Monat. Augenheilk.* **52**, 819.
Robertson (1915). *J. Biol. Chem.* **22**, 233.
Rona and György (1913). *Biochem. Z.* **48**, 278.
Rona and Takahashi (1913). *Biochem. Z.* **49**, 370.
Ruszynák (1921). *Biochem. Z.* **114**, 23.
Wessely (1908). *Arch. Augenheilk.* **60**, 1, 97.

XII. THE ABSORPTION SPECTRUM OF CHOLESTEROL AND ITS BIOLOGICAL SIGNIFICANCE WITH REFERENCE TO VITAMIN D. PART I.

PRELIMINARY OBSERVATIONS.

By ISIDOR MORRIS HEILBRON, EDWARD DAVID KAMM and RICHARD ALAN MORTON.

From the Department of Organic Chemistry, University of Liverpool.

(*Received December 6th, 1926.*)

It has been fully proved by various investigators that although cholesterol itself exhibits no antirachitic properties prior to irradiation with ultra-violet light, these are developed on comparatively short exposure to these rays. This brings the reaction within the sphere of photochemistry and since photochemical changes do not occur without the absorption of light and since it follows from the Grotthus-Draper law that only the rays absorbed are active, it would immediately become a matter of urgency to ascertain whether cholesterol showed well-defined absorption bands in the ultra-violet. The hitherto published results on this aspect of the problem are essentially preliminary in character. Hess and Weinstock [1925] observed that when cholesterol was irradiated by ultra-violet rays a change in the absorption spectrum occurred. The activated material absorbed light of certain wave-lengths to a less degree than ordinary cholesterol, a difference in the absorption over the entire range of wave-lengths (integrated) being detected by the use of a thermopile and galvanometer set. Schlutz and Ziegler [1926], carrying the matter further, observed that carefully recrystallised cholesterol, melting at 148·5° or very near that point, showed selective absorption "of wave-lengths between 294–296 $\mu\mu$ and 279–294 $\mu\mu$ with a great deal of general absorption beyond 294 $\mu\mu$." The absorption bands, which were evidently very shallow, could not be detected in alcoholic solution but showed up in ether or chloroform. Moreover, the bands were only observed in the first crop (five fractions collected) from a fractional crystallisation from alcohol of cholesterol (M.P. 148·5°) which had previously been recrystallised seven times from the same solvent.

As these results are not wholly consistent with the idea of ready photochemical change in the cholesterol molecule, we deemed it advisable to undertake a still more extensive and quantitative study of the absorption spectrum of this compound.

The results now to be described were obtained using a Hilger quartz spectrograph, rotating sector-photometer, iron-nickel arc arrangement. The arc (5 amps., 105 volts) was situated at a distance of 132 cm. from the absorbing solution. In our first experiments a sample of carefully purified cholesterol (from brain), M.P. 148·5°, kindly supplied by Prof. Drummond,

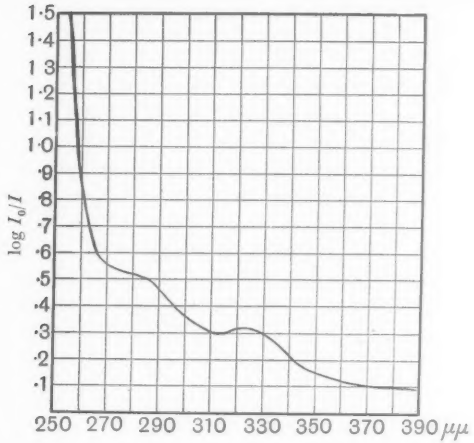

Fig. 1. Cod-liver oil film approx. $\frac{1}{10}$ mm. thickness.

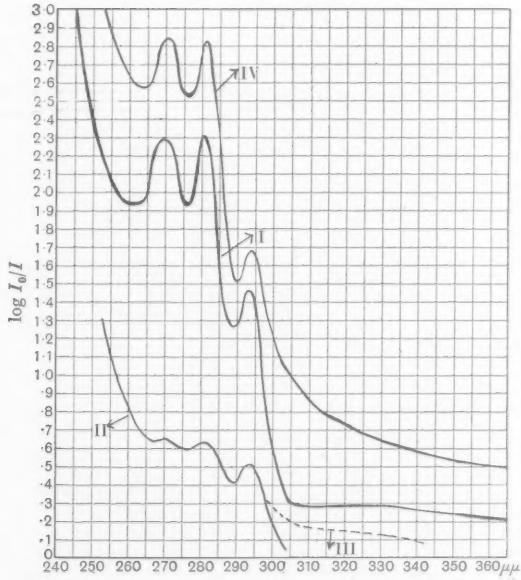

Fig. 2. I. Cholesterol (2½ g. from 200) 2 g. in 50 cc. ether, 4 cm. cell, fresh.
II. Drummond's cholesterol from brain—our ordinary purified 148·5° cholesterol.
III. Solution II after taking 1 plate (*i.e.* after standing 1 hour).
IV. Cholesterol (2½ g. from 200) 2 g. in alcohol, 4 cm. cell, fresh.

was employed and showed clearly the very shallow bands recorded by Schlutz and Ziegler [1926]. In addition it gave indications of an extended region of absorption on the ultra-violet side (Fig. 2, curve II). We next employed a sample of cholesterol obtained from cod-liver oil (M.P. 148·5°) and recrystallised by us three times. The absorption curve coincided exactly with that obtained in the previous case. An examination of the curves seemed to us to indicate that the observed curve was made up of two separate curves, one of which we regard as being due to cholesterol itself and the other to the material which becomes active after absorbing light energy.

With the idea of developing and testing this point of view, the unused portion of this cholesterol was once more recrystallised from ethyl acetate, the least soluble 2 g. being again examined spectrographically. It was found that this fraction gave a higher extinction, a higher definition for the bands and made the existence of a third band near 269 $\mu\mu$ certain (Fig. 3, curve III).

As these preliminary results indicated the presence of some foreign material in cholesterol to which the selective absorption could be attributed, we next undertook fractional crystallisation of 200 g. of our crude material. After one recrystallisation from ethyl acetate (1500 cc.) 50 g. were obtained, M.P. 147·5–148° (Charlottenberg certificated thermometer), and this portion was again dissolved in the same volume of ethyl acetate and the solution allowed to crystallise slowly. The portion which crystallised out (25 g.) melted sharply at 148·5°, the melting point being, if anything, fractionally higher than that of the pure specimen of brain cholesterol obtained from Prof. Drummond, which was at the same time re-determined as a check. Two further crystallisations of this material were carried out:

(*a*) the 25 g. were crystallised from 600 cc. ethyl acetate, yielding 5 g. of a less soluble portion;

(*b*) this latter portion was again dissolved in ethyl acetate (120 cc.), yielding after cooling 2·5 g. of material melting at 148·5°.

It will be noted that as the fractional crystallisation proceeded, the solubility of each successive fraction of residual substance decreased to a marked degree and that whereas the crude product was soluble to an extent of 10 % in ethyl acetate at room temperature, the final fraction which represents approximately 1 % of the original material was only soluble in this solvent under the same conditions to an extent of 2 %. It is thus clear that a substance differing in solubility from ordinary cholesterol may be concentrated by fractional crystallisation, but these results are only attained if an adequate quantity of material is originally taken.

A spectrographic examination of this highly concentrated product now showed that in this material the extinction coefficient had increased to approximately four times that of the purest material previously obtained[1] and

[1] We desire to record our thanks to Dr O. Rosenheim and Mr T. A. Webster who have now tested biologically this preparation and report that it is three to four times as active as a sample of the non-concentrated product. This shows remarkable agreement with the spectrographic results. [February 2, 1927. I. M. H.]

that the definition of all three bands was very much improved (Fig. 2, curves I, IV). Consistent results were obtained when the spectrograms were taken in either alcohol or ether solution.

With the idea of still further concentrating our "*X*-material," a fresh crystallisation was carried out. In this case we started from 2000 g. of the crude cholesterol, obtaining after one crystallisation from ethyl acetate 450 g. of less soluble material (M.P. 147·5–148°). This was repeatedly crystallised from large volumes of ethyl acetate yielding successively the following less soluble fractions: 117 g., 43 g., 6 g. This last portion was further crystallised from dilute alcohol, yielding 5 g. of material the absorption curve of which is reproduced on Fig. 5, curve I. The extinction coefficient has again increased beyond the highest point previously recorded, but, on the other hand, the bands, rather contrary to our expectation, have lost somewhat in definition.

An important fact regarding this highly concentrated material is that its melting point is not only definitely lower than that of purified material isolated from the less concentrated portions, but is indefinite over a range 146·5–149°. This result is quite consistent with our assumption of an "*X*-compound" in cholesterol. Moreover, this substance is almost entirely accumulated in the least soluble portion, for, on working up the filtrate from which the 43 g. portion was crystallised, a product was obtained of definitely higher melting point (148·3°) which nevertheless only showed very faint selective absorption, while the material (M.P. 148·5°) obtained by concentration of the filtrate from the previous crystallisation (117 g.) followed by careful fractionation showed no selective absorption whatsoever (Fig. 5, curve II).

A full chemical investigation of this least soluble fraction has just been started. While still in the early stages and awaiting confirmation, the results so far obtained certainly indicate the presence of a substance which has a slightly higher carbon content, is not precipitated with digitonin and does not show the typical Liebermann reaction for cholesterol.

It is of some interest to record that the addition of sodium ethoxide to an alcoholic solution of cholesterol (brain cholesterol from Prof. Drummond) favours increased definition to the band at 269 $\mu\mu$ at the expense of the other bands, whereas addition of hydrogen chloride causes the appearance of a new band at 318 $\mu\mu$. The latter band is not far removed from the second band of cod-liver oil first recorded by Schlutz and Ziegler [1926] and now confirmed by ourselves (Fig. 1). The significance of these results is not at the moment ripe for discussion.

Irradiation of cholesterol.

When cholesterol is irradiated in the solid state between quartz plates for 10 minutes at a distance of 6 inches from a quartz mercury lamp which had been in use for some time, almost complete disappearance of selective absorption is noted (Fig. 4, curve III). A sample of the brain cholesterol irradiated by Prof. Drummond in an atmosphere of nitrogen gave a smooth curve

showing only general absorption (Fig. 3, curve I). Moreover, all samples of cholesterol irradiated for periods longer than 10 minutes in the solid state or in alcoholic or ethereal solution (Fig. 3, curve II) resulted in the formation of a product exhibiting no signs of selective absorption. In the case of the least soluble fraction obtained from the 2000 g. fractionation, the absorption spectrum after irradiation (Fig. 5, curve III) although showing no bands has nevertheless a very high extinction coefficient.

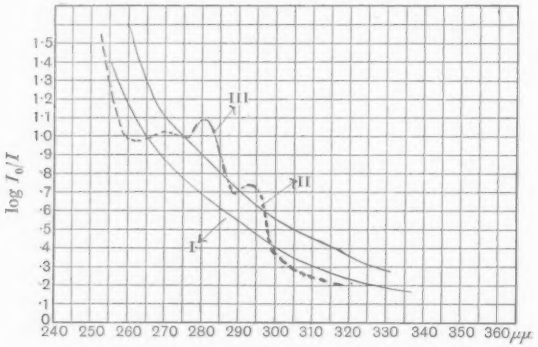

Fig. 3. I Drummond's irradiated cholesterol 2 g. in 50 cc. ether solution, 4 cm. cell.
II. Cholesterol irradiated in ethereal solution 2 g., 50 cc., 4 cm. cell.
III. Ordinary 148·5° cholesterol (from cod-liver oil) before irradiation.

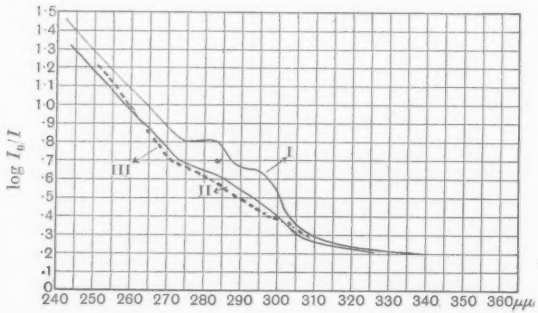

Fig. 4. I. 4 g. ordinary 148·5° cholesterol in 250 cc. alcoholic solution, 10 cm. cell, fresh.
II. Ditto after 2 hours.
III. Same cholesterol after 10 minutes' irradiation in solid state.

Finally we have found it impossible again to observe any of the characteristic bands in irradiated material which has subsequently been recrystallised. We have also confirmed the observation of Schlutz and Ziegler [1926] that a solution of cholesterol in alcohol or ether after standing four hours no longer showed the bands (Fig. 4, curves I and II).

Discussion.

The facts that purified cholesterol exhibits selective absorption before irradiation, and that the bands gain in definition and show increased extinction when the material is fractionally crystallised, all point to the presence of an "*X*-substance" in the original compound. Since the bands disappear on irradiation, with concomitant appearance of vitamin potency, it would seem that there is definite evidence for a correlation between absorption bands and a vitamin precursor. Various lines of investigation combine to show that the precursor is different from cholesterol itself. In the first place, the quantity of antirachitic material produced does not increase beyond a definite limit by prolonging the period of irradiation. This would not be expected if the cholesterol itself were being transformed.

Secondly, as already mentioned, cholesterol recovered from irradiated material no longer shows selective absorption.

Thirdly, Hess, Weinstock and Sherman [1925] failed to prove that deactivated cholesterol could acquire antirachitic potency by renewed irradiation.

Cholesterol becomes active when irradiated in solution—only in those solvents transparent to ultra-violet radiation of the wave-lengths covered by the bands. In acetone (Fig. 6, curve *d*), which effectively absorbs these rays, no activation should occur. This inference is strikingly confirmed by the fact recorded without comment by Hess, Weinstock and Sherman [1925], that irradiation in acetone confers no antirachitic potency.

Two possibilities must not be neglected in interpreting these results: firstly, it is quite conceivable that a material very rich in the vitamin precursor should undergo photochemical change very readily, even to the point of militating against its detection by spectrographic methods. Secondly, cholesterol itself may consist of a mixture of inactive stereoisomerides (compare Anderson and co-workers [1926] on sitosterol) which might differ in absorption spectra. Stereoisomerides frequently exhibit parallel curves differing only in extinction. Either of these alternatives would account for the apparent loss of definition in the bands shown by the end-fraction of the most concentrated material (Fig. 5, curve I).

We must accept, therefore, that cholesterol itself shows only general absorption and that in those fractions which exhibit selective absorption another substance is present. Fig. 6 is a purely geometrical illustration of the type of curve which would result in such a case. It will be seen (*a*) that the resulting summation curve is not dissimilar to the curves actually obtained in our concentrated cholesterol; (*b*) that the ultra-violet component of the band showing a triplet structure necessarily appears in a less well-defined manner than the longer wave component.

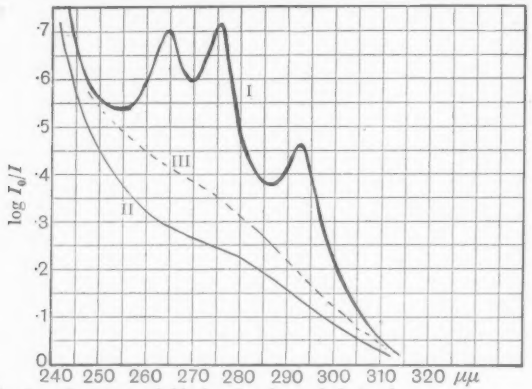

Fig. 5. I. Least soluble fraction from cholesterol.
II. Cholesterol after removal of active compound.
III. Irradiated fraction.

These curves are not strictly comparable with Fig. 1 but curve I in this figure corresponds with greater absorption than curve IV, Fig. 2.

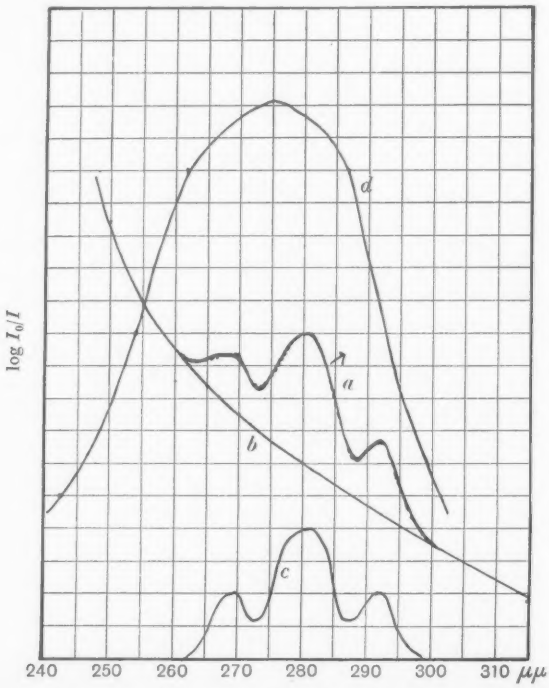

Fig. 6. (*a*) is a purely geometrical illustration of the effect of superposing selective absorption (*c*) on general absorption (*b*). (*d*) shows the absorption band of acetone on an arbitrary scale.

SUMMARY.

1. Ordinary purified cholesterol contains another compound in small quantity which can be accumulated in the least-soluble fraction.

2. This substance shows well-defined absorption bands at 293 $\mu\mu$, 280 $\mu\mu$, and 269 $\mu\mu$, while cholesterol itself has only general absorption.

3. These bands disappear on irradiation with ultra-violet light with concomitant appearance of antirachitic potency.

4. It is obvious that the unknown substance is closely connected with the vitamin D precursor.

We desire to express our thanks to Prof. J. C. Drummond and Mr H. J. Channon, of the Biochemical Department of University College, London, for providing us with brain cholesterol, to Messrs. Joseph Nathan and Co., Ltd. (Proprietors of Glaxo), for the large quantities of cod-liver oil cholesterol necessary for these experiments, and to the Food Investigation Board of the Department of Scientific and Industrial Research for a grant which has enabled the work to be carried out.

REFERENCES.

Anderson and co-workers (1926). *J. Amer. Chem. Soc.* **48**, 2972, 2976, 2987.
Hess and Weinstock (1925). *J. Biol. Chem.* **64**, 193.
Hess, Weinstock and Sherman (1925). *J. Biol. Chem.* **66**, 145.
Schlutz and Ziegler (1926). *J. Biol. Chem.* **69**, 415.

[Note added February 2, 1927.] R. Pohl (*Nachrichten der Gesellschaft der Wissenschaften zu Göttingen, Mathematisch-Physikalische Klasse*, 1926) communicated on December 10, 1926, a paper on the absorption spectrum of antirachitic cholesterol. A monochromator with double spectral resolution was employed in conjunction with a photoelectric photometer. The presence of three absorption bands which disappear on irradiation is confirmed and the absorption curve for inactive cholesterol is found to be quite smooth. The photoelectric method, using monochromatic light, thus establishes our view that no change occurs during the measurement of absorption spectra by the photographic method, and our results are fully confirmed.

XIII. THE FEEDING OF XANTHOPHYLL TO RATS ON A DIET DEFICIENT IN VITAMIN A.

By STANLEY GORDON WILLIMOTT and THOMAS MOORE.

From the Biochemical Laboratory, Cambridge.

(*Received December 6th, 1926.*)

Although the theory of the identity of vitamin A with one of the yellow plant pigments has been abandoned [Drummond and Coward, 1920], most of the experimental work leading to this conclusion has been based upon carotene, while xanthophyll appears to have received less direct attention. This is somewhat surprising in view of the fact that xanthophyll is present in plant tissues in excess of carotene, but is probably accounted for by the well-known fact that vitamin A accompanies carotene rather than xanthophyll in the phase test. Drummond [1919] fed crystalline carotene to rats in the proportion of 0·003 % of the total diet and found it to be ineffective as a source of vitamin A.

Stephenson [1920] removed the carotene of butter fat by adsorption with charcoal without affecting its content of vitamin A.

The evidence is not so complete in the case of xanthophyll. Rosenheim and Drummond [1920] studied the effect of a xanthophyll preparation on rats, but as this material was impure and an indication of growth was obtained, the results were rightly considered inconclusive. On the other hand, Palmer and Kempster [1919] were able to remove xanthophyll from the diet of growing chicks without adversely affecting their health, powers of reproduction, or the virility of their offspring through two generations. The tissues of these birds and their eggs were devoid of carotene and xanthophyll.

Signs of returning interest in the carotenoid pigments are indicated in the paper of Drummond, Channon and Coward [1925]. These authors found that vitamin concentrates prepared from the unsaponifiable fraction of cod-liver oil are always more or less yellow coloured, a fact which led them to consider the possibility of carotenoid pigments being involved. Lycopin and carotene were thus tested by these investigators and found to be inactive as a source of vitamin A, whilst a feeding experiment with xanthophyll was not undertaken as their material was not pure. It therefore seemed of interest to prepare a pure specimen of crystalline xanthophyll and to study its effect when fed in known amount to rats on a diet deficient in vitamin A compared with that of a potent source of this vitamin such as cod-liver oil.

Experimental.

The crystalline xanthophyll was prepared from young nettle leaves by the method of Jörgensen and Stiles [1917] with certain modifications. Air-dried powdered nettle leaves were sieved so as to remove stalks and midribs and placed in the hot room at 40° for 3 days. The powder was then quickly reground, transferred to a large desiccator and dried over sulphuric acid *in vacuo* for a further 4 days. In the preliminary extraction with acetone 2 litres of solvent were used for each 500 g. of nettle powder as the filtrate still appeared considerably coloured after the 1½ litres recommended by Jörgensen and Stiles had been added. It was also found advantageous to recover the solvent held by the material after filtration under reduced pressure by expression in the hand press. By so doing more than a litre of extract was recovered from 1500 g. of the original material. Otherwise the method of Jörgensen and Stiles was closely followed. The crude xanthophyll was recrystallised three times from methyl alcohol, a little water being added to assist crystallisation and the flask placed in the refrigerator.

The yield was found to be little more than a third of that stated by these authors for the crude product. Allowing for the fact that our yield refers to the pure product, it is possible that there is a difference in xanthophyll content between the very young nettles we used as our material and the mature plant usually employed. The crystals were placed in an evacuated desiccator shaded from the light. They melted at 172° and gave the usual coloured solutions in organic solvents and answered the colour tests. Under the microscope the xanthophyll appeared as reddish yellow prisms.

Feeding test.

With the small quantity of crystalline xanthophyll available it was not possible to feed the substance to more than two albino rats. These animals were "run out" on the usual basal diet of this laboratory, vitamin D being supplied by direct irradiation for 10 minutes daily at a distance of 2 feet from the mercury vapour lamp. After their weights had become constant xanthophyll was administered at the rate of 3 mg. per head per day in the form of pellets. The pellets were made up with a small quantity of the basal mixture containing the correct dosage and were stored for the feeding period in a well-evacuated desiccator shaded from the light. These precautions are emphasised by the recent work of Schertz [1925], who finds that in the dry state at room temperature, xanthophyll is oxidised even more readily than carotene both in light and darkness. One pellet per day was fed by hand to each of the animals in advance of the basal ration, and very soon they were found to consume their dosage without objection. After a preliminary rise, both animals showed a marked decrease in weight which was not due to lack of appetite as the food intake was normal. Eventually both animals died and autopsies revealed the usual signs of vitamin A deficiency. Two control

animals fed 16 mg. of cod-liver oil in liquid paraffin gave a satisfactory growth response within 12 days, while both experimental animals died from vitamin A deficiency after 20 days' feeding with xanthophyll. It was therefore concluded that xanthophyll is ineffective in a daily dose of 3 mg. as a source of vitamin A.

SUMMARY.

Pure crystalline xanthophyll prepared from nettle leaves cannot be identical with vitamin A.

Our thanks are due to Sir F. G. Hopkins.

REFERENCES.

Drummond (1919). *Biochem. J.* **13**, 81.
Drummond and Coward (1920). *Biochem. J.* **14**, 668.
Drummond, Channon and Coward (1925). *Biochem. J.* **19**, 1065.
Jörgensen and Stiles (1917). *New Phytol.* Reprint No. 10.
Palmer and Kempster (1919). *J. Biol. Chem.* **39**, 299, 313.
Rosenheim and Drummond (1920). *Lancet*, i, 862.
Schertz (1925). *J. Agric. Res.* **30**, 575.
Stephenson (1920). *Biochem. J.* **14**, 715.

XIV. THE TYROSINASE-TYROSINE REACTION.

VI. PRODUCTION FROM TYROSINE OF 5:6-DIHYDROXYINDOLE AND 5:6-DIHYDROXYINDOLE-2-CARBOXYLIC ACID—THE PRECURSORS OF MELANIN.

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From the Department of Physiology, University of Manchester.

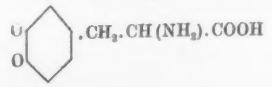
(*Received December 9th, 1926.*)

In the last communication of this series [Raper, 1926] it was shown that if a solution of the red pigment which is produced from tyrosine by tyrosinase is allowed to decolorise under suitable conditions, then 3 : 4-dihydroxyphenylalanine may be isolated from the solution. It was pointed out also that in the solution obtained by this method, the immediate precursor of melanin is present and that it possesses a structure in which the nitrogen is no longer in the amino form.

In the present paper an account is given of the identification of this precursor of melanin and the light it throws on the chemical changes involved in its production from tyrosine. It has not been possible to isolate the precursor itself because it is too easily oxidised, but its methyl derivative has been isolated in crystalline form and satisfactorily identified.

When the red solution obtained from tyrosine by the action of tyrosinase, as described previously [Raper, 1926], is allowed to decolorise *in vacuo* or in presence of sulphurous acid and then, after concentration in an atmosphere of CO_2, is methylated by means of methyl sulphate in an atmosphere of hydrogen, from the reaction product two crystalline methylated products can be obtained. One of them is a feeble base and the other an acid. The former is obtained principally when the red substance has been allowed to decolorise *in vacuo* and the latter when decolorisation takes place in the presence of sulphurous acid. Both products give colour reactions characteristic of indole derivatives.

It was postulated in the paper already mentioned that the 3 : 4-quinone of phenylalanine (I) is formed prior to the production of the red substance in the tyrosinase-tyrosine reaction; and in attempting to determine the structure of the indole derivatives to which the red substance gives rise, it had to be considered whether in the formation of the indole ring the N of the amino group of the phenylalanine quinone attached itself in the 2 or 6 position in the benzene nucleus. Union in the 6 position would produce



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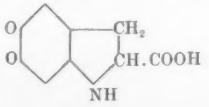
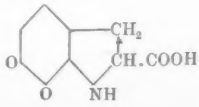


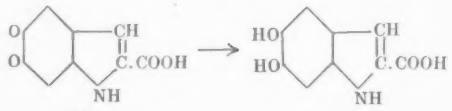
II



III

the 5 : 6-quinone of dihydroindole-2-carboxylic acid (II) and in the 2 position, the 6 : 7-quinone of dihydroindole-2-carboxylic acid (III). Further oxidation of II and III would result in the formation of the quinones of the corresponding indole carboxylic acids.

From the work of Jones and Robinson [1917] on the orientation of substituted catechol ethers it appeared to be most probable that the 5 : 6-quinone of indole-2-carboxylic acid would be formed, and it was suspected therefore that the methylated indole derivative with acid properties which was isolated from the products of oxidation was 5 : 6-dimethoxyindole-2-carboxylic acid, the quinone having undergone reduction to the corresponding dihydroxy-derivative in presence of sulphurous acid. It was also apparent that by loss of CO_2 from this acid or from the quinone with subsequent reduction, 5 : 6-dihydroxyindole would be obtained and that the feeble base isolated after methylation was therefore likely to be 5 : 6-dimethoxyindole. Since 5 : 6-dimethoxyindole and its carboxylic acid had not been previously prepared they were therefore synthesised[1] and found to be identical in all respects with the two indole derivatives isolated from the oxidation products. This being established it is of interest to discuss the probable formula of the red substance, its mode of formation and the changes it undergoes when it becomes decolorised. The provisional formula for the red substance given previously [Raper, 1926] must now be considered to be very unlikely in view of the fact that in the presence of sulphurous acid it gives rise to 5 : 6-dihydroxyindole-2-carboxylic acid (V).



IV V

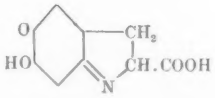
One way of explaining this transformation is to assume that the red substance is the 5 : 6-quinone of indole-2-carboxylic acid (IV) and that on reduction by sulphurous acid it yields the corresponding dihydroxy-compound. If this be the case then the decolorisation of the red substance which takes place in the absence of oxygen must be accompanied by a reduction as well as a loss of CO_2 since the main product obtained is 5 : 6-dihydroxyindole.

This process is slower than the decolorisation brought about by sulphurous acid, and since unchanged tyrosine is present in the solution it could be explained as being brought about at the expense of the tyrosine, which would be oxidised.

Alternatively, the red substance may be considered to be the 5 : 6-quinone of dihydroindole-2-carboxylic acid (II) and to undergo slow autoreduction with the loss of CO_2, thus producing 5 : 6-dihydroxyindole. This however

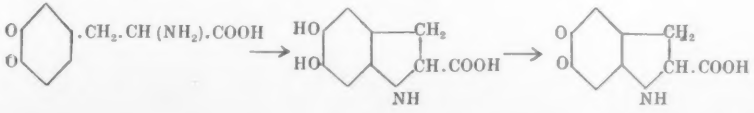
[1] The synthesis will be described elsewhere.

would render the decolorisation of the red substance in presence of sulphurous acid more difficult to understand, since on reduction it would yield 5 : 6-dihydroxydihydroindole-2-carboxylic acid, whereas the compound isolated after methylation is not a dihydroindole derivative. That a dihydroindole derivative is actually produced but subsequently oxidises in the air in the process of extraction subsequent to methylation is possible but unlikely. On the other hand the sulphurous acid may act simply as a catalyst and accelerate the autoreduction, which, proceeding more rapidly, would be less likely to result in the loss of CO_2 and would thus give rise to the production of the dihydroxyindole carboxylic acid. When the autoreduction is not catalysed by SO_2 and consequently proceeds relatively slowly there is more opportunity for the loss of CO_2 and dihydroxyindole is produced. Another alternative formula for the red compound which gives it a *para-* instead of an *ortho*-quinonoid structure is the following:



This offers the same difficulty as II, namely, that it does not explain the production of an indole derivative rather than a dihydroindole derivative in presence of sulphurous acid. On the other hand, as in the case of (II) the difficulty disappears if it be assumed that the action of the sulphurous acid is catalytic and favours autoreduction. It might also be expected to have a yellow rather than a red colour which is more characteristic of the *ortho*-quinonoid grouping.

From these considerations it appears most probable that the red compound is the 5 : 6-quinone of dihydroindole-2-carboxylic acid and that this substance undergoes autoreduction and loss of CO_2 slowly at the ordinary temperature and more rapidly on heating with production of 5 : 6-dihydroxyindole. In the presence of sulphurous acid, however, the autoreduction proceeds more rapidly and consequently the loss of CO_2 is much less marked. This results in the production mainly, therefore, of 5 : 6-dihydroxyindole-2-carboxylic acid. The experimental results show that in presence of sulphurous acid this is almost the only product produced, only traces of the base being obtained. In the absence of sulphurous acid, however, the base forms almost the entire product which can be isolated, only traces of the acid being obtained. If these views be correct, then the formation of the red substance from the 3 : 4-quinone of phenylalanine may be represented as follows:



The first stage may be considered as analogous to the formation of an anilino-quinone, the second is brought about by the oxidation of the dihydroxydihydroindole carboxylic acid by the enzyme. This is probably rapid since it is analogous to the oxidation of 3 : 4-dihydroxyphenylalanine to the corresponding quinone which occurs in an earlier stage of the tyrosinase reaction and is known to take place rapidly.

The presence of dihydroxyphenylalanine in small amount along with the indole derivatives is accounted for partly by its being an intermediate between tyrosine and the quinone of phenylalanine, and partly by the reduction of the latter by sulphurous acid when this is used to accelerate the decolorisation of the red substance. It has already been suggested by the author that 3 : 4-dihydroxyphenylalanine is the first product produced from tyrosine when it is oxidised by tyrosinase and its isolation and identification as a product of the reaction, together with other evidence put forward in the last communication of this series, rendered this very probable. But it seemed to be important, in order to place the matter beyond doubt, to demonstrate that the red substance produced when tyrosinase acts on 3 : 4-dihydroxyphenylalanine will undergo transformation to yield the same indole derivative that tyrosine produces under the same conditions. This has now been done. When the red substance, obtained from 3 : 4-dihydroxyphenylalanine by the action of the enzyme, was allowed to decolorise *in vacuo*, it was possible to isolate 5 : 6-dimethoxyindole from the resulting products by the same procedure that was used in the case of tyrosine.

These results place on a more secure basis the hypothesis first advanced by Happold and Raper [1925] that tyrosinase produces *ortho*-quinones from those phenols which bring about deamination when acted upon by the enzyme in presence of an amino-acid. This view has been extended to oxidases in general which will act upon substances containing the catechol grouping by Szent-Györgyi [1925], who has also made it the basis of a theory as to the nature of oxidases. Experiments in progress in the author's laboratory indicate very clearly that tyrosinase acting on phenol and *p*-cresol produces *o*-quinones, for it has been found that when the enzyme is allowed to act on either of these phenols in the presence of aniline, considerable amounts of the anilino-quinones are obtained. The complete results of these experiments will be published shortly.

It is presumed from the results described in this paper that melanin production occurs by the oxidation in the air of 5 : 6-dihydroxyindole and to a lesser extent of 5 : 6-dihydroxyindole-2-carboxylic acid. These substances have not been synthesised, but there is no doubt that they would be extremely easily oxidised in the air and especially in slightly alkaline solution—the optimum condition for melanin production from tyrosine. Attempts to remove the methyl groups from dimethoxyindole or its carboxylic acid have been fruitless as might be expected, since this can only be done in acid solution, under which conditions both the indole and its carboxylic acid yield tarry

products [*vide* Perkin and Rubenstein, 1926]. Under slightly alkaline conditions the oxidation of the traces of dihydroxyphenylalanine which are found amongst the products of action of the enzyme would contribute to melanin formation also [Bloch and Schaaf, 1925].

The formation of melanin from dihydroxyindole and its carboxylic acid is of considerable interest from the point of view of the normal production of melanin in pigment cells and in melanotic tumours. From time to time observations have been made which indicated that melanin on destructive decomposition yielded cyclic compounds containing nitrogen which give the pine-shaving reaction. This has been taken by some to mean that melanins were possibly formed from blood pigment. Evidence against this view has been collected by von Fürth [1912] and it is now not generally accepted. On the other hand it has led to the view (von Fürth) that tryptophan might be the mother-substance of melanin in pigment-producing cells. The isolation from the urine in a case of melanotic sarcoma of a substance which gave indole reactions has also given support to this view [Eppinger, 1910] and especially so since administration of tryptophan increased the amount of this substance excreted, whereas tyrosine did not. On the other hand, Bloch and Schaaf [1925] have advanced the view that melanin is formed from 3 : 4-dihydroxyphenylalanine by a specific enzyme "dopa oxydase," and a structure for the pigment is given in which its formation is based on the production of the 3 : 4-quinone of phenylalanine, two molecules of which are represented as condensing with one molecule of the 3 : 4-quinone of phenylacetaldehyde.

It would appear from the observations recorded in this paper that the cyclic group containing nitrogen is already present in the precursors of melanin and that it is likely to persist when melanin has been formed by oxidation of these precursors in the air or by means of tyrosinase. Whether, however, the melanin obtained from tyrosine by oxidation with tyrosinase is identical with that found in pigment cells cannot be yet considered as proven. It is on the whole unlikely, since Abderhalden and Guggenheim [1907, 1908] have shown that peptides containing tyrosine will also produce melanin when oxidised by the enzyme and it is likely that the peptide linkage will persist in the final product. It does however seem probable that the reactions leading to the formation of the indole nucleus from tyrosine, or peptides containing it, as described in this paper, are an essential preliminary in the natural production of melanin.

Experimental.

The enzyme used in the following experiments was prepared from the mealworm (*Tenebrio molitor*) as described previously by the author [Raper, 1926].

I. *Production of* 5 : 6-*dimethoxyindole from tyrosine.*

1 g. of tyrosine is dissolved in 500 cc. hot water and the solution diluted to 2 l. in a large stoppered bottle.

The temperature is adjusted to 26–30° by cooling or warming as required and 100 cc. of the enzyme preparation are added. The p_H is now adjusted to between 6 and 6·5 by the cautious addition of 1 % acetic acid. Oxygen is bubbled through the solution for 1–2 minutes and the bottle is stoppered and well shaken. About every half-hour oxygen is bubbled through the liquid and it is frequently shaken. The solution quickly becomes red and in 2 to 4 hours most of the enzyme has been precipitated. To ensure complete precipitation 10 cc. of 1 % acetic acid are added and after standing for a few minutes the solution is filtered quickly, using several large funnels. The deep red filtrate is placed in a large filter flask and the flask evacuated. The liquid is well shaken to remove as much dissolved oxygen as possible and hydrogen is then admitted. It is now again evacuated and re-filled with hydrogen. The red liquid is allowed to stand at the ordinary temperature under hydrogen until the red tint has been lost and replaced by a pale brown. 10 cc. of a saturated solution of SO_2 are now added to minimise oxidation in the succeeding operations. The solution is now filtered to remove a small amount of brown deposit and placed in a flask from which it can be displaced gradually and continuously to a large distillation flask while at the same time being kept under hydrogen. It is then concentrated by vacuum distillation in a stream of CO_2 until it measures about 20 cc. The reaction products in this solution together with unchanged tyrosine are now methylated in an atmosphere of hydrogen. The flask is detached and fitted with a reflux condenser, inlet and outlet tubes for hydrogen and a dropping funnel. The flask is evacuated and filled with hydrogen, this process being repeated once to ensure complete absence of oxygen.

With a slow stream of hydrogen passing through the flask, the methylation is carried out by first adding 10 cc. of 20 % NaOH, shaking gently till the contents of the flask are all in solution, and then adding 3·5 cc. dimethyl sulphate in two portions (2·5 and 1 cc.). After each addition the flask is vigorously shaken. It is now heated on the water-bath for an hour, cooled and the brown alkaline solution extracted three times with ether. The ether extract is washed with a small amount of water. On removal of the ether by distillation, from 100 to 150 mg. of the crystalline dimethoxyindole have usually been obtained. It was recrystallised from a small volume of alcohol and was pure after two crystallisations, M.P. 154–5°. Mixed with synthetic 5 : 6-dimethoxyindole the M.P. remained unchanged.

Analysis. Found: C, 67·6 %; H, 6·2 %; N, 8·3 %.
Calculated: C, 67·8 %; H, 6·2 %; N, 7·9 %.

If the alkaline solution resulting from the methylation after extraction with ether is acidified to Congo red and again shaken out several times with ether, traces of an acid are obtained which on crystallisation from benzene containing a small amount of acetone melts at 202–3° with evolution of gas. This acid is therefore 5 : 6-dimethoxyindole-2-carboxylic acid (see next section).

A trace of the base dissolved in alcohol and mixed with a few drops of *p*-dimethylaminobenzaldehyde in alcohol gave a violet colour on adding a drop of 20 % HCl. Addition of a trace of sodium nitrite changed the colour to a deep crimson. The substance gave a reddish violet colour with sodium nitroprusside and caustic soda which was changed to a pure blue on adding acetic acid. Mixed with glyoxylic acid and run on to concentrated sulphuric acid the substance gave a red colour at the junction of the two liquids.

II. *Production of* 5 : 6-*dimethoxyindole-2-carboxylic acid from tyrosine.*

The procedure to be carried out up to the stage at which the solution of the red pigment is obtained is the same as described in the previous section. Instead of keeping the red solution under hydrogen, it is mixed with 50–60 cc. of a saturated solution of SO_2 and allowed to stand for 1 to 2 days in a stoppered bottle. The pale yellow solution is now concentrated *in vacuo* in a large distilling flask and when reduced to about 20 cc. is methylated in an atmosphere of hydrogen exactly as described above. The alkaline solution obtained after methylation is extracted with ether and usually yields a trace of crystalline substance which gives indole reactions and is presumably 5 : 6-dimethoxyindole. On acidifying and shaking out several times with ether a substance is obtained which is left as a vitreous residue after evaporation of the ether. It crystallises on allowing it to stand with water for a few hours and is crude 5 : 6-dimethoxyindole-2-carboxylic acid. The yield has usually been about 0·2 g. from each gram of tyrosine initially taken. It was purified by dissolving in the minimal volume of acetone and adding 15 volumes of hot benzene, filtering at once and allowing to stand. It crystallised in flattened, rhombic prisms, M.P. 202–3° with evolution of gas. Mixed with synthetic 5 : 6-dimethoxyindole-2-carboxylic acid the M.P. remained unchanged.

Analysis. Found: C, 59·8 %; H, 5·1 %; N, 6·6 %.
Calculated: C, 59·7 %; H, 5·0 %; N, 6·3 %.

In the *p*-dimethylaminobenzaldehyde reaction it gives a less intense violet colour than 5 : 6-dimethoxyindole. If it be heated above its M.P. until CO_2 evolution ceases and the residue then tested, the indole reaction is more marked and similar in all respects to that given by pure 5 : 6-dimethoxyindole. The acid or base dissolved in glacial acetic acid gives a yellow colour when one drop of nitric acid is added (brucine test).

III. *Production of* 5 : 6-*dimethoxyindole from* 3 : 4-*dihydroxyphenylalanine.*

1 g. dihydroxyphenylalanine prepared from *Vicia faba* according to the directions of Guggenheim [1913] was dissolved in 2 l. water, cooled to 27° and 70 cc. of the enzyme solution added. The p_H was adjusted to 6·5 and the solution saturated with oxygen. The solution rapidly became a deep red and in 10 minutes the enzyme had precipitated out. After half-an-hour 10 cc. of 1 % acetic acid were added and the solution was filtered. The red filtrate was

dealt with exactly as described in the first section. After methylation 136 mg. of the crude base were obtained. This was recrystallised twice from alcohol and had M.P. 154–5°. Mixed with synthetic 5 : 6-dimethoxyindole the M.P. remained the same. It gave the typical indole reaction with *p*-dimethylaminobenzaldehyde.

SUMMARY.

1. 5 : 6-Dihydroxyindole and 5 : 6-dihydroxyindole-2-carboxylic acid have been identified as products of the action of tyrosinase on tyrosine and are to be regarded as the immediate precursors of melanin.

2. 5 : 6-Dihydroxyindole is the chief product produced when the red pigment which is characteristic of the tyrosinase reaction undergoes intramolecular change. 5 : 6-Dihydroxyindole carboxylic acid is however produced principally when the red substance is allowed to decolorise in the presence of sulphurous acid.

3. 3 : 4-Dihydroxyphenylalanine also gives rise to 5 : 6-dihydroxyindole under the same conditions that result in its production from tyrosine. This confirms the previous suggestion that 3 : 4-dihydroxyphenylalanine is the first intermediate product in the tyrosinase-tyrosine reaction.

4. The formula previously given to the red pigment produced from tyrosine has been revised in view of the further evidence presented in this paper. It is probably the 5 : 6-quinone of dihydroindole-2-carboxylic acid.

5. The production of melanin is discussed.

I wish to thank Mr T. Kirkman for the preparation of the dihydroxyphenylalanine used in this work, and the Government Grant Committee of the Royal Society for a grant in aid of expenses. I wish also to express my appreciation of the kindness of my colleague, Prof. R. Robinson, for his assistance on several points in connection with the theoretical aspects of this work.

REFERENCES.

Abderhalden and Guggenheim (1907). *Z. physiol. Chem.* **54**, 331.
—— —— (1908). *Z. physiol. Chem.* **57**, 329.
Bloch and Schaaf (1925). *Biochem. Z.* **162**, 181.
Eppinger (1910). *Biochem. Z.* **28**, 181.
Von Fürth (1912). Probleme d. pathol. u. physiol. Chem. (Leipzig).
Guggenheim (1913). *Z. physiol. Chem.* **88**, 276.
Happold and Raper (1925). *Biochem. J.* **19**, 92.
Jones and Robinson (1917). *J. Chem. Soc.* **111**, 903.
Perkin and Rubenstein (1926). *J. Chem. Soc.* **130**, 357.
Raper (1926). *Biochem. J.* **20**, 735.
Szent-Györgyi (1925). *Biochem. Z.* **162**, 399.

XV. THE CONSTITUTION AND SYNTHESIS OF SPERMIDINE, A NEWLY DISCOVERED BASE ISOLATED FROM ANIMAL TISSUES.

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The presence of an unidentified base in the mother-liquors after the isolation of spermine phosphate from tissue extracts was originally observed by M. C. Rosenheim and O. Rosenheim during their early work on spermine and has already been reported [Dudley, Rosenheim and Rosenheim, 1924].

This base has been given the name *spermidine* since it not only occurs in association with spermine but also has been found to be structurally related to the latter substance. Its phosphate is much more soluble than that of spermine and crystallises from the 25 % alcoholic mother-liquor, after the removal of spermine phosphate, on increasing the concentration of alcohol to about 50 %.

The material employed in the present investigation was isolated by this procedure during the large scale preparation from ox pancreas of the spermine used in our recently published work on the constitution of the latter base [Dudley and Rosenheim, 1925; Dudley, Rosenheim and Starling, 1926]. The yield of spermidine phosphate is extremely small, being only one-tenth of that of spermine phosphate, namely about 2 g. from 100 kg. pancreas. Only 10 g. of spermidine phosphate were available but, aided by our recently acquired knowledge of the constitution of spermine, we were able to arrive at a probable constitutional formula for the base, and then to prove its correctness by synthesis.

The properties of spermidine are very similar to those of spermine. This is demonstrated in a general way by the fact that it accompanies spermine through all the stages of isolation from tissue extracts by the three methods already described [Dudley, Rosenheim and Rosenheim, 1924]. Like spermine it gives a pyrrole reaction, it appears in the lysine fraction on applying the method of Kossel and Kutscher, its phosphotungstate is insoluble in acetone, it is optically inactive, it does not reduce permanganate in faintly acid solution

and it yields the characteristic semen-like odour when a solution of its chloroaurate is treated with magnesium.

Analyses of its salts gave figures very close to those of the corresponding salts of spermine but indicated that the ratio of carbon to nitrogen in spermidine is lower than in spermine. The molecular weight of the *m*-nitrobenzoyl derivative of the base, considered in conjunction with the analyses, established the molecular formula of spermidine as $C_7H_{19}N_3$. In view of the striking similarity between spermidine and spermine it was considered probable, therefore, that spermidine might be α-(γ′-aminopropylamino)-δ-aminobutane, $NH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH_2$, a formula which is derived from that of spermine, whose structure we have shown to be

$$NH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.NH_2,$$

by the replacement of one of the terminal γ-aminopropylamino-groups by an atom of hydrogen. The correctness of this conjecture was proved by the following synthesis:

$C_6H_5O.CH_2.CH_2.CH_2.Br + NH_2.CH_2.CH_2.CH_2.CH_2.NH_2$
α-phenoxy-γ-bromopropane α:δ-diaminobutane

$\rightarrow C_6H_5O.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH_2$
α-(γ′-phenoxypropylamino)-δ-aminobutane

HBr

$\rightarrow Br.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH_2$
α-(γ′-bromopropylamino)-δ-aminobutane

NH_3

$\rightarrow NH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH_2$
α-(γ′-aminopropylamino)-δ-aminobutane

The final compound proved to be in all respects identical with natural spermidine, whose constitution is thus established.

Experimental.

Spermidine phosphate.

An equal volume of alcohol is added to the concentrated mother-liquor from the preparation of spermine phosphate [Dudley, Rosenheim and Rosenheim, 1924].

Spermidine phosphate crystallises from this solution in lustrous plates, usually contaminated with a small amount of spermine phosphate. The easily soluble spermidine phosphate is dissolved in the minimum requisite quantity of cold water and the spermine phosphate, which remains suspended in the solution, is filtered off. To the filtrate, warmed on the water-bath, alcohol is added until a faint permanent turbidity is produced. On allowing the solution to cool spermidine phosphate crystallises. It can be recrystallised from a hot 20 % solution in water. The crystals so obtained are similar in appearance to those of cholesterol and entirely different from the characteristic spermine phosphate.

Heated in a melting-point tube spermidine phosphate begins to shrink at 150° and melts at 207–9° to an opaque fluid which begins to froth at 218–20° without discolouring.

Analysis. 0·2944 g. lost 0·0463 g. H_2O at 108°

Found: H_2O, 15·73 %
Calc. for $(C_7H_{19}N_3)_2.3H_3PO_4$, $6H_2O$: H_2O, 15·61 %
0·2341 g. (dry) gave 0·1336 g. $Mg_2P_2O_7$
0·16155 g. (dry) gave NH_3 = 16·72 cc. $N/10$ H_2SO_4 (Kjeldahl)
Found: P, 15·89 %; N, 14·49 %
Calc. for $(C_7H_{19}N_3)_2.3H_3PO_4$: P, 15·92 %; N, 14·38 %

Spermidine picrate.

This salt was prepared by adding excess of sodium picrate solution to a solution of spermidine phosphate. The picrate, which is sparingly soluble in water, when recrystallised from this solvent is obtained as lemon-yellow needles. Under the microscope these are seen to be composed of long thin laminated plates. Under certain conditions of concentration and rate of cooling the crystals formed are broader, being composed of the same thin laminae fused together and appearing to the naked eye as thin plates. Spermidine picrate melts at 210–12° to an oily liquid which almost immediately decomposes and froths slowly up the tube.

Analysis. 0·0473 g. (dried at 102°) gave 0·0924 g. nitron picrate
Found: picric acid, 82·68 %
Calc. for $C_7H_{19}N_3.3C_6H_3O_7N_3$: picric acid, 82·58 %

Spermidine hydrochloride.

3·55 g. spermidine picrate were treated on the water-bath with a mixture of 45 cc. absolute alcohol, 5 cc. concentrated hydrochloric acid and 2·5 cc. water. The picrate dissolved in the hot solution and the hydrochloride crystallised in needles on cooling. The crystals were redissolved by heating and 25 cc. acetone were added. The hydrochloride crystallised from the cooled solution and was filtered off, washed with acetone and dried in a vacuum desiccator: yield 1·04 g. On recrystallisation from a mixture of 50 cc. absolute alcohol and 8·5 cc. concentrated hydrochloric acid it was obtained in glistening thin plates, containing no water of crystallisation.

Spermidine hydrochloride is only very slightly hygroscopic; it is quite stable in a relatively dry atmosphere but slowly deliquesces when the humidity is high.

Analysis. Dried at 100°. 0·1396 g. gave 0·1717 g. CO_2 and 0·1055 g. H_2O
" 0·1331 g. gave NH_3 = 15·75 cc. $N/10$ H_2SO_4 (Kjeldahl)
" 0·1258 g. gave 0·2121 g. AgCl
Found: C, 33·54 %*; H, 8·46 %; N, 16·53 %; Cl, 41·71 %
Calc. for $C_7H_{19}N_3.3HCl$: C, 33·01 %; H, 8·64 %; N, 16·50 %; Cl, 41·85 %

* Slightly high owing to incomplete reduction of oxides of nitrogen during the combustion.

Spermidine chloroaurate.

0·1 g. spermidine hydrochloride was dissolved in 7 cc. water. On addition of 5 cc. 10 % "gold chloride" solution an immediate crystalline precipitate appeared. After standing at 2° for 2 hours the chloroaurate was filtered off and recrystallised from 6 cc. 1 % hydrochloric acid.

It forms thin, lustrous, golden yellow plates; M.P. 220–2° with decomposition.

When a more dilute solution is allowed to stand for some time at 2° the salt crystallises in long thin needles with the same melting-point as the plates.

Analysis. 0·1833 g. dried at 100°, gave 0·0930 g. Au.

Found: Au, 50·73 %
Calc. for $C_7H_{19}N_3.3HAuCl_4$: Au, 50·72 %

m-*Nitrobenzoylspermidine.*

To 0·15 g. spermidine hydrochloride, dissolved in 6 cc. *N* NaOH, 0·9 cc. molten *m*-nitrobenzoyl chloride was added and the mixture was vigorously stirred in a small beaker. As soon as the reaction of the liquid became nearly neutral 2 cc. *N* NaOH were added and this addition was repeated as necessary. A sticky white mass was formed which slowly became more solid. When the substance became plastic it was pressed out to a thin cake, the mother-liquor was poured off and 2 cc. *N* NaOH were added. After standing overnight the crude *m*-nitrobenzoyl derivative had become friable and was broken up to a powder, filtered off and washed with water: yield (air-dried) 0·336 g.

It was suspended in 20 cc. absolute alcohol. When warmed on the water-bath the solid melted to an oil which then slowly dissolved. To the filtered solution 20 cc. water were added, the turbid solution was warmed until clear and set aside for a day. The *m*-nitrobenzoyl derivative had then crystallised in aggregates of thick needles. These were filtered off and washed with 50 % alcohol: yield (air-dried) 0·298 g. The substance melted at 102° to a turbid liquid which slowly frothed up the tube without charring at higher temperatures. On recrystallising under the same conditions it behaved in the same manner and the melting-point was unchanged. It was then dried in a vacuum desiccator over H_2SO_4. On warming the substance (0·28 g.) with 25 cc. absolute alcohol the oil which formed at first rapidly changed to a white solid which went very slowly into solution. From this solution, after standing overnight, the substance crystallised in balls of radiating needles. These were collected and again recrystallised from 20 cc. absolute alcohol. On heating the substance with alcohol it no longer formed an oil but remained solid and dissolved very slowly. 0·2155 g. was obtained which melted at 148–50°. In all probability the low-melting product originally obtained was a hydrate.

Molecular weight determination (Rast).

0·0042 g. was dissolved in 0·0378 g. camphor. Δ 7·5°, M.W. 592.
Calc. for $C_7H_{16}N_3$ $(CO.C_6H_4.NO_2)_3$: M.W. 592.

Analysis. 0·1590 g. (dried for 2 days *in vacuo* over H_2SO_4) gave 20·8 cc. N_2 at 20° and 728 mm. (Dumas).

Found: N, 14·37 %
Calc. for $C_7H_{16}N_3$ $(CO.C_6H_4.NO_2)_3$: N, 14·20 %

Spermidine forms a chloroplatinate and a mercurichloride which are very soluble. It also yields a crystalline phenylisocyanate derivative, but these compounds are not suitable for purposes of identification.

SYNTHESIS OF SPERMIDINE.

α-(γ'-Phenoxypropylamino)-δ-aminobutane hydrobromide.

This substance was isolated as a by-product from the preparation of α:δ-bis(γ'-phenoxypropylamino)-butane hydrobromide used in the synthesis of spermine [Dudley, Rosenheim and Starling, 1926]. The mother-liquor from the recrystallisation of the latter substance was concentrated and, after adding alcohol, was poured into 10 volumes of ether. The white crystalline precipitate (5·8 g.) was recrystallised from 96 % alcohol, separating in thin rectangular plates, M.P. 271–3°.

Analysis. 0·1950 g. gave 0·1896 g. AgBr.
0·3648 g. gave NH_3 = 19·6 cc. *N*/10 H_2SO_4 (Kjeldahl)

Found:	Br, 41·38 %; N, 7·52 %
Calc. for $C_{13}H_{24}ON_2Br_2$:	Br, 41·67 %; N, 7·30 %

α-(γ'-Bromopropylamino)-δ-aminobutane hydrobromide.

4·6 g. of the above phenoxy-compound were heated in a sealed tube at 100° for 12 hours with 25 cc. HBr solution (Sp. G. 1·7). On shaking the clear solution with ether to remove phenol a crystalline substance was precipitated; this was filtered off and washed with alcohol and ether. After three recrystallisations from absolute alcohol 2·4 g. of prismatic needles were obtained: M.P. 234–5°, which proved to be the required substance.

Analysis. 0·0664 g. gave 0·1014 g. AgBr.

Found:	Br, 64·97 %
Calc. for $C_7H_{18}N_2Br_3$:	Br, 64·69 %

α-(γ'-Aminopropylamino)-δ-aminobutane picrate (synthetic spermidine picrate).

1·5 g. of the above bromo-compound were heated in a sealed tube at 100° for 4½ hours with 19·4 g. of an alcoholic solution of ammonia (16 % by weight). The reaction mixture was then evaporated *in vacuo*. The white solid remaining was dissolved in water, potassium hydroxide was added to give a concentration of about 50 % and the solution was then steam-distilled until the distillate gave only a faint cloud on adding picric acid solution. The first fraction of the distillate (200 cc.) was boiled for 5 minutes to expel ammonia. The whole distillate was then neutralised with hydrochloric acid and evaporated to small bulk. Addition of sodium picrate solution precipitated a picrate which, after recrystallisation, was found to be identical with the picrate of natural spermidine. From this picrate the *hydrochloride*, *chloroaurate* and m-*nitrobenzoyl derivative* were prepared. These compounds displayed exactly the same properties as those of natural spermidine. Mixed samples of the corresponding derivatives of the synthetic and natural bases showed no depression of melting points.

Analyses of synthetic spermidine salts.

0·0448 g. picrate gave 0·0872 g. nitron picrate

Found:	picric acid, 82·38 %
Calc. for $C_7H_{19}N_3.3C_6H_3O_7N_3$:	picric acid, 82·58 %

0·1558 g. chloroaurate gave 0·0784 g. Au.

Found:	Au, 50·32 %
Calc. for $C_7H_{19}N_3.3HAuCl_4$:	Au, 50·72 %

As the problem of identifying minute amounts of spermidine may arise in the future a few remarks in this connection may not be out of place. In extracts of animal tissues the base will usually be found in association with spermine. The only feasible way of separating these two substances is to convert them into phosphates and then to remove the very insoluble spermine salt. It is essential to separate the two bases completely in this way before preparing the picrates, since it appears to be impossible to separate the two picrates by recrystallisation. There is a characteristic difference between the melting-points of the picrates: that of spermidine forms an oil at 210–12° which decomposes almost immediately, whilst that of spermine gradually darkens until it becomes black, softening and decomposing at 248–50° without first melting. The *m*-nitrobenzoyl derivative of spermidine is valuable for identification of the base since a molecular weight determination by Rast's method [1923] requires no more material than is used in an ordinary melting-point determination and since there is a considerable difference between the molecular weights of the corresponding derivatives of spermine and spermidine. The preparation and analysis of a chloroaurate is not decisive on account of the very slight differences in melting-point and gold content of these salts of the two bases.

It was pointed out in our communication on the structure of spermine [Dudley, Rosenheim and Starling, 1926] that this base is the first example of a naturally occurring compound of the type $NH_2.R.NH.R'.NH.R.NH_2$ and at the same time the first derivative of trimethylenediamine definitely identified among natural products. It is interesting to find the trimethylenediamino-group in a second naturally occurring base, spermidine, which is the first example found in nature of the closely related type

$$NH_2.R.NH.R'.NH_2.$$

It seems reasonable to suspect that trimethylenediamine itself may be detected among the bases extracted from fresh tissues; in this connection the fact may be recalled that tetramethylenediamine has already been definitely identified in the mother-liquors after the isolation of spermine from yeast [Dudley, Rosenheim and Rosenheim, 1924].

It is hardly necessary to state that the chemical procedures incidental to the isolation of spermine and spermidine cannot have produced artificially the latter from the former. The two bases undoubtedly exist together in animal tissues, and their close chemical relationship strongly suggests that they may be metabolically related.

SUMMARY.

1. A hitherto undescribed base, having the molecular formula $C_7H_{19}N_3$, has been isolated from extracts of animal tissues.

2. In occurrence and constitution it is allied to spermine and has therefore been given the name *spermidine*.

3. The structural formula of spermidine, proved by synthesis, is

$$NH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH_2,$$

i.e. α-(γ'-aminopropylamino)-δ-aminobutane.

REFERENCES.

Dudley and Rosenheim (1925). *Biochem. J.* **19**, 1032.
Dudley, Rosenheim and Rosenheim (1924). *Biochem. J.* **18**, 1263.
Dudley, Rosenheim and Starling (1926). *Biochem. J.* **20**, 1082.
Rast (1923). *Z. physiol. Chem.* **126**, 111.

XVI. STUDIES ON THE GROWTH OF YEAST.

III. A FURTHER STUDY ON THE INFLUENCE OF VOLUME OF MEDIUM EMPLOYED.

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(*Report to the Medical Research Council.*)

(*Received December 18th, 1926.*)

In a previous communication [1925, 1] I have recorded the growth of single-cell cultures of yeast in various volumes of medium. At the time of publication of those experiments I calculated that in the series of volumes employed I had included the different volumes of medium per yeast cell inoculated which were used by Liebig and Pasteur. I subsequently found that owing to an error in all my calculations the volumes which I had actually studied were not in cubic millimetres as recorded, but in thousandths of a cubic millimetre. I had therefore only studied concentrations of yeast equal to and greater than those used by Pasteur, and even in the largest volumes I had not employed such a low concentration of yeast as did Liebig. On this account the experimental evidence of the communication did not justify any conclusions as to the causes of the differences in growth observed by Pasteur and Liebig. The more general conclusions concerning the relationship between volume and growth are obviously not affected by any alterations in the units in which the volumes were recorded. In the experiments detailed below I have extended the range of volumes to include that which corresponds with the concentration of yeast employed by Liebig.

Experimental.

The experimental details will be found in the previous paper [Peskett, 1925, 1]. The yeast used was the same strain of *Saccharomyces cerevisiae*, and the cells whose growth was studied in these experiments were washed before inoculation by the technique described elsewhere [Peskett, 1925, 2]. The stock and parent cultures were grown on medium 5. All cultures were grown at 25°. Medium 1 consisted of the basal salt mixture plus 50 g. per litre commercial cane sugar; in medium 2 the latter was replaced by recrystallised cane sugar, and medium 5 was composed of medium 2 plus 10·0 cc. per litre yeast extract.

Volumes. In Exp. 1 the volumes were approximately 5·0 and 0·5 mm.^{3}. If a yeast cell can be considered as a sphere of 10 μ diameter, *i.e.* about

5×10^{-7} mm.^{3} volume, then single cells in these volumes would correspond to concentrations of 0·01 mm.^{3} and 0·1 mm.^{3} of yeast per 100 cc. of medium respectively. These concentrations are approximately those used by Liebig and Pasteur, as will be seen from the discussion in my previous communication [1925, 1, p. 471].

In Exp. 2 the volumes were 39·7 mm.^{3} and 0·04 to 0·01 mm.^{3}, *i.e.* roughly 10 times larger than Liebig's, and 10 times smaller than Pasteur's per yeast cell; in addition three cultures were studied in which the volumes were intermediate.

Preparation of the cultures. For use in Exp. 1 a silica capillary pipette was calibrated to deliver 5·0 mm.^{3} of mercury determined by weighing. A suspension in medium 1 of washed yeast cells was prepared of such a dilution that 5·0 mm.^{3} of it could be distributed in 10 drops of approximately equal diameter, a few of which would contain a single cell. Thus hanging-drops were prepared of about 0·5 mm.^{3} volume, some of which contained a single cell.

The 5·0 mm.^{3} drops of medium 1 were measured from the capillary pipette on to slides, each of which had a deep circular depression about 13/16 inch in diameter. The drops were each inoculated with a single washed cell transferred by means of a capillary pipette from hanging-droplet preparations in medium 2 which had been prepared previously. Particular care was taken to search these droplets thoroughly, and in every case only those were used which contained one cell beyond all doubt. Immediately after inoculation the cultures were covered by a cover-slip sealed in position with paraffin wax. The under surface of the cover-slips had been treated before sterilisation with a commercial preparation to prevent fogging owing to condensation of water. This did not come in contact with the cultures and therefore did not vitiate the results.

The error in measuring the drops in Exp. 1 was fairly considerable. The pipette delivered 4·9 mm.^{3} of mercury $\pm$ 0·07 mm.^{3}. In measuring medium this error must at least have doubled owing to wetting of the surface by the fluid. Thus the probable error was at least 3 % for the larger drops, and it may have increased up to 10 % for the smaller though every effort was made to keep the diameters of the latter constant within 0·1 mm.

In Exp. 2 the largest volumes were measured (1 drop) from a Dreyer pipette which delivered 252 drops of medium 1 per 10·0 cc. The volumes of the other drops were calculated from measurements of their diameter and depth. The largest and intermediate drops were set up and inoculated as in Exp. 1, the smallest were prepared in the usual manner from a suspension of washed cells in medium 1.

Every precaution was taken to prevent contamination of the cultures.

Observation and bacterial control. The cultures were examined at intervals of about 4 hours, and the growth recorded. In the case of the larger drops it was often necessary to warm the cover-slip to dispel the thin film of water which had condensed thereon, in order to get clear definition of the cells under

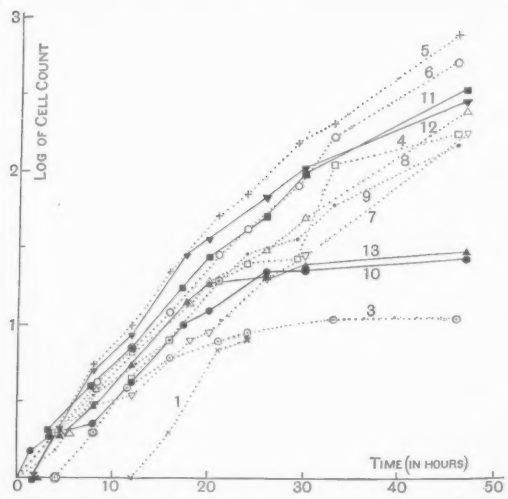

Fig. 1. Growth of washed single cells in medium composed of recrystallised salts and commercial cane sugar.

Volume (mm.³)	
5·0	———
0·5	. . . . .

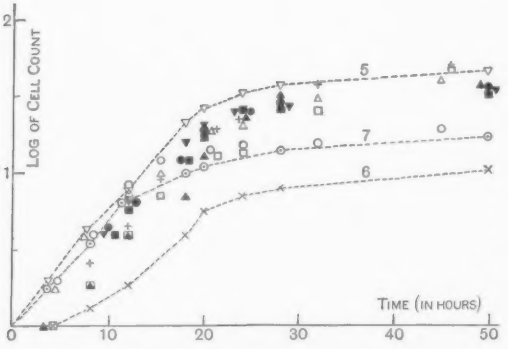

Fig. 2. Growth of washed single cells in medium composed of recrystallised salts and commercial cane sugar.

Volume (mm.³)	
40	▲ ▼ ■ ●
1·4	▽ - - - - ▽
1·3	⊙ - - - - ⊙
1·0	× - - - - ×
0·04	□ +
0·02	○
0·008	△

the microscope. If this precaution was observed the cells were counted quite easily, using a low-power objective and high-power ocular.

In Exp. 2 great difficulty was experienced in locating the single cells in the largest drops: in three cases out of the five they were not found until the third observation (10 hours).

At the end of the growth period all the cultures were examined for contamination, either by fixing and staining *in situ*, or by transferring a loopful to nutrient agar, which was then incubated at 25° for 2 days followed by a further 2 days at 37°. In the latter case smears were prepared from the agar slopes and stained and examined in the usual manner. Any infected cultures were excluded from the results.

Results.

In each experiment I have recorded on a graph the logarithm of the number of cells present at each observation. Buds were considered as fractions, computed on the basis of the relationship between their diameters and those of the cells from which they were growing. The graphs are shown in Fig. 1 (Exp. 1) and Fig. 2 (Exp. 2).

In Table I, I have compared in each experiment the total number of bacteria-free cultures which were prepared with the number of these which actually showed growth.

Table I.

	Exp. 1		Exp. 2	
Volume	Total	Growing	Total	Growing
Largest	6	4 (66 %)	5	4 (80 %)
Intermediate	—	—	4	3 (75 %)
Smallest	12	8 (66 %)	6	4 (66 %)

Discussion.

It is clear from Table I that the incidence of mortality among the cultures is fairly constant in spite of the differences in volumes. In Exp. 2 the greatest mortality occurred in the smallest volumes, possibly on account of relatively greater evaporation from the smallest drops. It seems unlikely therefore that Liebig's failure to obtain growth was due to death of the cells inoculated.

In Exp. 1 the behaviour of all the cultures up to the 20th hour was very uniform, and closely comparable to that observed in Exp. 2. After the 20th hour there was a marked retardation of growth in two of the cultures in the larger volumes (Nos. 10 and 13). At the same time one of the cultures in the smaller volumes was retarded to an even greater degree (No. 3), and in another case (No. 1) there was some retardation at first. In the latter case the drop touched the surface of the slide at the 24th hour, after which growth could no longer be observed. At the conclusion of the experiment I could only count a total of 13 cells on the slide and cover-slip, but I have omitted this observation from the graph on account of its uncertainty. With the exception

of these four cultures, uniformity of growth was observed from the 20th hour to the end of the experiment.

In Exp. 2 the largest variations in growth were observed in the three cultures in intermediate volumes (Nos, 5, 6, 7). I am inclined to attribute these variations to the relatively large degree of evaporation which must have occurred, since the drops were placed in large depression slides similar to those used for the largest drops. In this connection it is perhaps significant that the greatest variation occurred in the smallest drop (No. 6). Apart from these variations the growth of all the cultures in this experiment was strikingly uniform.

In Exp. 2, although the conditions were similar to those of Exp. 1, the final growth obtained was far less. I cannot at the moment account for this difference, but it does not appear to affect the point at issue.

Thus, not only in volumes approximately 10 times larger or smaller than those used by Liebig and Pasteur, but also in volumes comparable with those which these observers employed per yeast cell inoculated, the growth of single cells of yeast appears to be uniform. Liebig's failure to obtain growth does not therefore appear to have been due to any effect of volume *per se* on the vitality or growth of the yeast.

It would seem more probable that the absence of "bios" from Liebig's cultures affords the true explanation. For, as the following experiment shows, it is possible to obtain growth of a single washed yeast cell in a volume as large as 4·0 cc.

Growth of single yeast cells in large volumes.

In the following experiment I have studied the growth of single cells in volumes of 0·08, 1·0 and 4·0 cc. Hanging-droplet preparations of single washed cells in medium 2 were prepared, and the cells transferred by means of a capillary pipette as in the experiments reported above. The 0·08 cc. cultures were grown in depression-slides, the 1·0 and 4·0 cc. in test tubes of suitable size. The medium used was medium 5. Growth was determined after 48 hours at 25° by a cell-count in a Bürker counting-chamber. In the case of the 0·08 cc. cultures the cells were washed off the slide with 1·0 cc. of medium 2 into a small test tube, the medium being delivered drop by drop from a calibrated Dreyer pipette. Examination of the slide after this process showed that very little yeast remained on it.

Table II.

Exp. 3. Inoculum one cell. Thousands of cells present in each culture after 48 hours at 25° are recorded in the third column.

Volume cc.	Culture No.	Growth $\times 10^3$	Error $\times 10^3$
0·8	1	914	25
0·8	2	892	25
1·0	3	551	25
4·0	4	712	60
4·0	5	1215	80
4·0	6	922	130

The results are recorded in Table II. The distribution of cells was rather uneven, and the figures for the counts were low (about 1 for each large square of 1/25 mm.^{2}). There is thus considerable error in these figures. In the last column of the table I have recorded the probable error of the counts, calculated by taking extreme values of the figures obtained for each row of 12 squares.

All the cultures in this experiment were free from contamination. With the exception of No. 3, the growth of all the cultures was approximately uniform, and there was no evidence of any inhibitory effect in the 4·0 cc. cultures. The figures are too few to justify any comparison of the effects of the different volumes, especially as the experiment has not been repeated. It is clear, however, that provided that the composition of the medium is suitable a single yeast cell can increase a millionfold in 48 hours when inoculated in a volume of medium 8,000,000,000 times its own. The growth of single cells in a medium composed of recrystallised cane sugar and salts (medium 1) has never in my experience exceeded 20-fold, and has generally been about 10-fold in 48 hours. These observations are in accord with those of Hansen [1879] and Kossowicz [1903]. The former inoculated a series of flasks with very small quantities of yeast, and, to obtain pure cultures, only employed those flasks which showed single colonies after incubation. But, as Lafar [1910] points out, cultures arising from a single cell can only be obtained from gelatin plates actually examined under the microscope, and this was the method subsequently adopted by Hansen [1883]. It cannot therefore be stated with absolute certainty that Hansen grew single cells in comparatively large volumes of fluid medium, but he certainly obtained growth from very small inocula. Kossowicz failed to observe multiplication, as judged by the naked eye, of single cells in media composed of salts and pure sugar, while large inocula ("viele Zellen") showed rich development under similar conditions. He was inclined to attribute the growth of the latter to "carry over." We must conclude therefore that failure of growth of small inoculations of yeast is due to deficiencies in the composition, and not to the volume, of the medium used. The explanation of the Liebig-Pasteur controversy as a result of differences in amount of "bios" present appears to be justified.

Summary.

1. The growth and vitality of single cells of yeast does not appear to be affected by the volume of medium employed, provided that the composition of the medium is suitable.

2. The differences of growth observed by Pasteur and Liebig cannot be ascribed to differences of the volume of medium used per cell inoculated, but were probably due to the presence of different amounts of "bios." This conclusion, although stated in a previous communication, was not justified by the results reported at that time.

In conclusion I desire to thank Prof. Peters for his interest and advice. I am indebted to the Medical Research Council for a part time grant, and to the Trustees of the Christopher Welch Fund for the purchase of apparatus.

REFERENCES.

Hansen (1879). *Compt. Rend. Lab. Carlsberg*, **1**, 49, 197. Cited by Lafar (1910).
—— (1883). *Compt. Rend. Lab. Carlsberg*, **2**, 13.
Kossowicz (1903). *Z. Landwirtsch. Versuchs. Oesterreich*, **6**, 731.
Lafar (1910). Technical Mycology, **2**, 219.
Peskett (1925, 1). *Biochem. J.* **19**, 464.
—— (1925, 2). *Biochem. J.* **19**, 474.

XVII. THE STOMACH OIL OF THE FULMAR PETREL (*FULMARUS GLACIALIS*).

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(*Received December 3rd, 1926.*)

In the course of an investigation on the suggested relationship between vitamin A and the arsenious chloride colour reaction of cod-liver oils [Rosenheim and Webster, 1926, 1], it seemed desirable to examine some other oils, differing essentially in their character and origin from cod-liver oil. Our attention was drawn by Sir Walter Fletcher to a communication by Malcolm [1926], in which the stomach oil of the Australasian mutton bird or white-headed Fulmar (*Oestrelata lessoni*) was stated to be a rich source of vitamin A. Purdy [1918] had previously suggested the body-fat of these birds as a substitute for cod-liver oil, and had found it effective in the treatment of bronchitic conditions and phthisis.

We were unable to obtain mutton bird oil in this country, as it appears to be produced commercially in small amounts only in certain islands near Tasmania and New Zealand. It seemed of interest therefore to examine the stomach oil of an arctic representative of the order Tubinares, the Fulmar petrel (*Fulmarus glacialis*), which corresponds most closely to the antarctic mutton bird. These birds breed in enormous numbers in St Kilda, and have extended their breeding colonies in recent years to an extraordinary extent in the Orkney and Shetland Islands [Coward, 1926]. They are purely oceanic wanderers, and seldom, if ever, come to land, except for the purpose of breeding. When disturbed on the nest, the bird ejects, as a defensive measure, with considerable force, some of the oil stored in the stomach.

We found that the stomach oil not only gives the arsenious chloride reaction to approximately the same degree as cod-liver oil, but that its content of growth-promoting vitamin A runs parallel to the colour value. The oil also contains the calcifying vitamin D.

A chemical examination showed that the oil is not a glyceride, but a "liquid wax," containing nearly 40 % of unsaponifiable matter. The latter appears to consist mainly of unsaturated higher alcohols of the same type as those found in sperm oil. The resemblance of the Fulmar stomach oil to that contained in the head cavity of the Spermaceti Whale (*Physeter macrocephalus*, L.) is so striking as to suggest a similarity in the origin and function of these oils which will be discussed later.

Experimental.

Through the kindness of Capt. S. R. Douglas we were able to examine a nearly full-grown bird, which was shot in August, at the end of the breeding season, in the island of Yell, Shetland. The bird weighed 800 g. and its wing measure was 290 mm. (adult birds = 318–330 mm.). The proventriculus was found to be completely filled with oil, which was collected by ligaturing the organ and making an incision. The oil, 50 cc. in amount, was perfectly clear, deep amber-coloured, and of a penetrating musty odour, resembling that of arctic plankton[1].

The natural untreated oil was used throughout this investigation, and was protected against light and air which rapidly bleach it.

The thick layer of blubber beneath the skin yielded 72 % of a nearly colourless fluid oil. This, as well as the fat of the liver and muscular tissue, was also examined for comparison with the stomach oil. In order to avoid destruction of the vitamin during the preparation of the fats, the tissues were immediately put into 5 % KOH solution. On standing at room temperature for 24 hours, the proteins dissolved and the fats were extracted with pure ether in a separating funnel. The ether extract was washed with water, dried with anhydrous Na_2SO_4 and concentrated in a current of nitrogen. The fats were finally dried *in vacuo*.

Colour tests.

Quantitative determinations of the blue colour given in Rosenheim and Drummond's $AsCl_3$ test [1925], were carried out by means of Lovibond's colorimeter. The results were identical with those obtained by the more convenient modified method, introduced by Carr and Price [1926], in which a chloroform solution of $SbCl_3$ is used instead of undiluted $AsCl_3$. We have, however, recently met with oils of low vitamin A content in which the modified method indicated the absence of vitamin A, whilst the results of the colorimetric "arsenic" test agreed with the biological growth test. This lack of sensitiveness in the "antimony" method is due to the dilution of both the oil and reagent and necessitates a control test with undiluted $AsCl_3$ in all apparently negative cases.

Table I.

	Arsenic and antimony reaction			Fearon reaction		
	Mg. oil in 1 cc. reagent	Units of blue		Mg. oil in 1 cc. reagent	Units of red	
		Measured	C.L.O. = 1*		Measured	C.L.O. = 1*
Cod-liver oil	20	10·0	1·0	20	20	1·0
Fulmar stomach oil	20	8·0	0·8	20	11·0	0·55
,, subcutaneous fat	100	2·0	0·04	100	12·0	0·12
,, muscle fat	20	2·2	0·22	20	1·5	0·07
,, liver fat	0·5	16·0	64·0	20	Nil	Nil

* C.L.O. = 1 signifies the numbers of blue or red units given by 20 mg. oil, taking cod-liver oil = 1.

[1] We had occasion to notice the smell of plankton also in the stomach contents of a whale (*Balaenoptera rostrata*), which had been alive for 3 days after having been stranded on the East Coast.

In Table I the results obtained with an active Newfoundland cod-liver oil are included for comparison, together with those of Fearon's [1925] colour test.

It will be seen that the Fulmar stomach oil possesses four-fifths of the colorimetric value of the standard cod-liver oil, whilst that of the subcutaneous and muscle fat is much less. On the other hand, the liver fat is 64 times as strong as cod-liver oil. The amount of this liver fat available was not sufficient for a growth test, but we found liver fats of other animals of equally high colour value, the growth-promoting power of which we are at present investigating.

Fearon's test has no relation to vitamin A, as we have previously shown, and the results are interesting only in confirming our statement that the reaction is negative in the case of liver fats of birds [Rosenheim and Webster, 1926, 1].

Feeding tests.

(1) *Vitamin A.* The technique described by Drummond, Coward and Handy [1925] was employed, in which a constant supply of vitamin D (irradiated cholesterol) is given throughout the experiment. In the course of this and similar investigations we found it convenient to correlate the dose of the oil to be tested with its colorimetric value by taking as the standard the number of blue units (Lovibond's scale) given by 10 mg. and 20 mg. of cod-liver oil in the colour test. Under the conditions adopted 20 mg. of Newfoundland oil equal 10 units of blue, and administered daily restore normal growth, *i.e.* induce a weekly increase in body-weight of 8–10 g. In the present work the Fulmar oil was administered in daily doses of 10 mg. and 20 mg., representing 4 and 8 units of blue respectively. In our experience the animal test, as at present developed, is not sensitive enough to differentiate variations of ± 2 units of blue, *i.e.* deviations of ± 4 mg. from the average dose of 20 mg. The results are represented in Fig. 1.

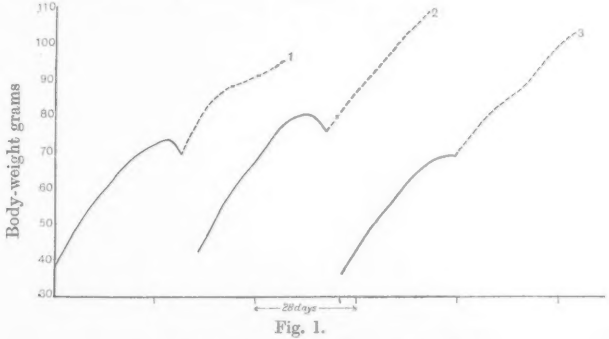

Fig. 1.

– – – – 1 = 10 mg. Fulmar oil = 4 Blue units.
– – – – 2 = 20 mg. „ „ = 8 Blue units.
– – – – 3 = 20 mg. cod-liver oil = 10 Blue units.

It will be seen that 20 mg. (= 8 blue units) of the Fulmar stomach oil produced growth of the same order as the same quantity of the standard cod-liver oil (= 10 blue units). Xerophthalmia had developed in all cases during the preparatory period, which cleared up rapidly on administration of the oil.

The growth tests made with mutton bird oil by Malcolm [1926] are open to criticism, since a constant supply of vitamin D had not been given. The statement "that mutton bird oil is one of the richest known natural sources of vitamin A" requires modification, as a good Newfoundland cod-liver oil induces similar growth increments in the doses given by Malcolm.

(2) *Vitamin D.* The technique previously described [Rosenheim and Webster, 1926, 2] was used. A daily dose of 20 mg. gave partial protection, whilst 40 mg. completely protected the animals against rickets. The presence of vitamin D in mutton bird oil has up till now not been demonstrated.

Chemical examination.

The untreated stomach oil of the Fulmar is a clear, deep orange-red, non-drying oil which begins to solidify when cooled to 8–9°, becomes waxy at 5° and sets to a translucent solid at 0°. Table II contains the constants of the oil, determined by the standard methods, together with those of mutton bird oil[1] [Carter, 1921], a genuine sperm oil [Dunlop, 1908], and an average cod-liver oil.

Table II.

	Fulmar oil	Mutton bird oil	Sperm oil	Cod-liver oil
Specific gravity	0·884	0·884	0·880	0·925
Saponification value	122	120	122	185
Iodine value (Wijs)	118	130	70	168
Higher alcohols	37·7 %	38·4 %	41·2 %	0·5–1·5 %
M.P. of ditto	31·5°–32·5°	31°–31·5°	31·5°–32·5°	?
Free fatty acid	1·6	2·2	1·4	0·3–1·5
Cold test	8°–9°	6°	7°–9·5°	Variable

These figures justify the conclusion that the composition of the stomach oil of these representatives of the arctic and antarctic petrels is practically identical. Both oils differ fundamentally from cod-liver oil in their low specific gravity and their high percentage of unsaponifiable substances (alcohols) which characterise them as "liquid waxes." On the other hand, they both resemble sperm oil, their higher iodine value being due to the nature of the unsaturated fatty acids they contain.

The fatty acids were fluid at room temperature. In the elaidin test they solidified only partially, thus indicating the presence of a certain amount of oleic acid. Their mean molecular weight was 292 and the iodine value 156. The high iodine value points to the presence of highly unsaturated fatty acids and the bromination test was therefore carried out.

[1] The earlier analyses of mutton bird oil [Smith, 1911; Evers and Foster, 1920] are in general agreement with that by Carter [1921].

1·134 g. of the fatty acids gave 0·238 g. = 20·9 % of ether-insoluble bromides. The white substance did not melt when heated in a capillary to 200° and began to darken at 240°, turning black without fusing at 250°. The product, being insoluble in organic solvents, was purified by extraction at the boiling-point with ether and benzene, in which it was practically insoluble. The behaviour on heating remained unchanged, thus excluding the presence of the hexabromide of linolenic acid (M.P. 175°).

Analysis: 0·0620 g. gave 0·1038 g. AgBr. Found 71·3 % Br.

Calculated for $C_{22}H_{34}O_2Br_{10}$: 70·9 %

„ „ $C_{20}H_{32}O_2Br_8$: 67·8 %.

The substance is therefore the decabromide of clupanodonic acid, which has been found by Tsujimoto [1922] as a characteristic constituent of sardine oil and other fish oils. The amount of ether-insoluble bromides in sperm oil is only 1·1–2·3 % [Dunlop, 1908], and this fact explains the difference in the iodine value of Fulmar and sperm oil.

The fatty acids give Fearon's test, which is ascribed by Rosenheim and Webster [1926, 3] to aldehydic oxidation products of clupanodonic acid.

The alcohols. The unsaponifiable fraction of the oil is a deep ruby-red wax, which rapidly bleaches on exposure to light and air. The $AsCl_3$ reaction is given in great intensity by freshly prepared specimens. Liebermann's cholesterol reaction is positive and Fearon's reaction negative, as in the unsaponifiable fraction of cod-liver oil. The alcohols were recrystallised from light petroleum, filtered at a low temperature and were thus obtained as a pale yellow wax, melting at 32°, easily soluble in organic solvents. Their iodine value is 54, and on bromination a small amount of a white bromide (cholesterol bromide?) is obtained. A quantitative estimation, by means of digitonin, gave 2·4 % cholesterol, showing that the bulk of the alcohols is unsaturated. On acetylation with acetic anhydride the acetates were obtained as oils, which solidified at 0° and melted at 5°. The acetates formed a clear solution in acetic anhydride, indicating the absence of any considerable amount of hydrocarbons. We were unable to obtain any direct evidence for the presence of cetyl alcohol, which is assumed to constitute the bulk of the alcohols of mutton bird oil [Carter, 1921]. In their general character the alcohols of the Fulmar oil resemble the unsaturated alcohols of unknown constitution, which have been obtained from sperm oil by Lewkowitsch [1892]. Recently oleic alcohol has been isolated from the unsaponifiable fraction of arctic sperm oil by Tsujimoto [1925].

The similarity in composition of the two oils is further exemplified by the fact that we were able to isolate only 1·4 % of glycerol from Fulmar oil, and that sperm oil also yields not more than 1·3–2·5 % glycerol [Dunlop, 1908].

The lipochromes. The solution of the pigments in light petroleum, obtained on recrystallisation of the alcohols (see above) was subjected to the Kraus phase-test [Willstätter and Stoll, 1913]. The pigment was found to be entirely epiphasic, thus showing it to be related to, or identical with, carotene, and

proving the absence of xanthophyll. This was confirmed by the chromatographic analysis according to Tswett, in which the light petroleum solution passed completely through the chalk column without showing any xanthophyll layers. Spectroscopic examination also indicated the presence of carotene, although the second and third absorption bands were partially obscured by general absorption.

The pigment is easily absorbed by charcoal (norit) from an ethereal solution of the oil. The colourless filtrate, after removal of the ether, gave quantitatively the same amount of blue colour with $AsCl_3$ as the original oil. The presence of the lipochrome therefore does not interfere with the colour test.

Possible function of the stomach oil.

We were unable to find any previous reference in the literature throwing light on the presence of the large amounts of oil in the stomach of the Fulmar petrel, except that it appears to be peculiar to all the members of the order Tubinares [Challenger Reports, 1882]. Since the oil, like other waxes, is resistent to hydrolysis by lipoclastic enzymes, it might be assumed to represent the indigestible residue of fatty foodstuffs. Such an assumption has indeed been made by Carter and Malcolm [1926], who were unable, however, to find any evidence for the presence of similar waxes or their alcohols in the fats of fishes, or any form of marine life, which might serve as food for these birds.

The resemblance of this fluid wax in its chemical character to that secreted by the "preen" glands of terrestrial birds suggests a different explanation. The rump gland wax of geese and ducks contains, like the stomach oil of petrels, about 40 % of alcohols, in which the presence of octadecyl alcohol has been definitely established by Röhmann [1904]. This wax has been shown by Röhmann to be a true secretory product and its obvious use to the bird in preening is to protect the feathers against the action of water, for which it is better suited than the easily saponifiable fats.

We would suggest that the stomach oil of the petrels has a similar origin and fulfils a similar function in these marine birds, which owing to their environmental conditions require a larger supply of preening material than terrestrial birds. Such a suggestion is strengthened by the presence of a distinctive structural feature in the order Tubinares, *i.e.* "their prominent tubular nostrils and their bills which consist of several horny pieces, separated by deep grooves" [Godman, 1910]. Through these structures the oil may be distributed over the beak, thus facilitating the preening operation when the bird is resting on the sea[1]. Another peculiarity of the oil, shared by sperm oil, is its low viscosity, which according to our determination is 0·216 at body temperature (36°). This characteristic feature distinguishes it from animal

[1] It might be mentioned that the petrels are said to eject the oil "through their nostrils" when disturbed. Certain statements in the literature lend support to the suggestion that the secretory gland may have to be searched for near the nasal cavity, the proventriculus serving merely as a store for the secretion.

fats and renders easy the transference of the oil from the proventriculus to the beak and aids in its distribution over the feathers. The presence of unsaturated fatty acids and alcohols in the wax helps to keep it fluid at relatively low temperatures, a property especially useful to a bird whose life is spent in the arctic regions.

The above view is rendered still more likely when we consider the analogous composition and function of similar waxes in certain aquatic mammals, the cetaceans. The liquid wax, sperm oil, present in the head cavity of many whales is practically of the same composition as the Fulmar stomach oil (see above), whilst that of dolphins and porpoises appears to form an intermediate link between liquid waxes and blubber oils [Lewkowitsch, 1921]. In the whale a canal running along the whole length of the body to the tail communicates with the head cavity and, probably in a way at present not properly understood, enables the secretion to reach and to spread over the surface of the skin, thus protecting the animal against the action of the sea water [Röhmann, 1908].

This suggested explanation for the function of the stomach oil does not account for the presence in it of vitamins. It may be pointed out, however, that the oil which we examined came from a juvenile bird which had not yet left the nest. The food received from the parents would probably supply more than the necessary amount of fat-soluble vitamins and thus explain their presence in the stomach oil. The examination of the stomach contents of a non-breeding adult bird would be of interest in this respect, and we intend to examine this and other questions during the next breeding season.

Summary.

1. The stomach oil of the Fulmar petrel (*Fulmarus glacialis*) has been examined chemically and biologically. It gives the arsenious chloride reaction approximately to the same degree as cod-liver oil, and its content of vitamin A runs parallel to the colour value. The oil also contains vitamin D.

2. The oil is not a glyceride, but a liquid wax of similar composition to sperm oil.

3. An explanation for the origin and the function of the oil is suggested.

REFERENCES.

Carr and Price (1926). *Biochem. J.* **20**, 497.
Carter (1921). *J. Soc. Chem. Ind.* **40**, 220.
Carter and Malcolm (1926). *Trans. New Zealand Inst.* **56**, 647.
Challenger Reports (1882), **4**, *Pt XI, Anatomy of the Petrels.*
Coward (1926). The Birds of the British Isles (London).
Drummond, Coward and Handy (1925). *Biochem. J.* **19**, 1068.
Dunlop (1908). *J. Soc. Chem. Ind.* **27**, 63.
Evers and Foster (1920). *Pharm. J.* **51**, 100.
Fearon (1925). *Biochem. J.* **19**, 888.
Godman (1910). Monograph of the Petrels (London).
Lewkowitsch (1892). *J. Soc. Chem. Ind.* **11**, 134.

Lewkowitsch (1921). Chem. Techn. Oils, Fats and Waxes (London).
Malcolm (1926). *Trans. New Zealand Inst.* **56**, 650.
Purdy (1918). *Brit. Med. J.* i, 174.
Röhmann (1904). *Hofmeister's Beitr.* **5**, 110.
—— (1908). Biochemie (Berlin).
Rosenheim and Drummond (1925). *Biochem. J.* **19**, 753.
Rosenheim and Webster (1926, 1). *Lancet*, ii, 806.
—— —— (1926, 2). *Biochem. J.* **20**, 537.
—— —— (1926, 3). *Biochem. J.* **20**, 1342.
Smith (1911). *J. Soc. Chem. Ind.* **30**, 405.
Tsujimoto (1922). *Chem. Umschau*, **29**, 261.
—— (1925). *Chem. Umschau*, **32**, 125.
Willstätter and Stoll (1913). Untersuch. über Chlorophyll (Berlin).

XVIII. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

III. TIME-DILUTION CURVES AND ZONES OF ACTION.

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This paper, which is intended to amplify one already published [Ponder, 1926, 2] is concerned with the expression

$$\kappa t = \frac{1}{S-\psi S} \log \frac{c}{c-\psi x-x} \quad \text{.....(1),}$$

which, when applied to a population of cells whose resistances are distributed according to a normal frequency curve, describes in a very satisfactory manner the action of the simple haemolysins[1]. In the expression, c is the concentration of lysin acting on the cells, x the quantity used up in the reaction with the cells, κ a velocity constant, and t the time taken for the lysin to bring about any specified degree of lysis; S is a term relating to the surface of the suspension, while ψ denotes a probability which can vary between 0 and 1. The derivation of the expression is discussed in the paper quoted.

This paper falls into two parts. In the first part we shall examine the extent to which the expression (1) describes the time-dilution curves of various lysins, and in the second part we shall find values for ψ, and thence values for the size of the hypothetical "zone of action" surrounding the cells of the particular haemolytic systems considered, within which the reaction between the cells and the lysin may be imagined to proceed.

1. The time-dilution curves.

The time-dilution curve for any haemolysin is obtained by plotting the dilution of lysin against the time which it takes to bring about complete haemolysis of a certain number of cells. The technique is fully given in a previous paper [Ponder, 1926, 1] and papers quoted therein. In all the cases given below the suspension used was prepared by suspending the thrice washed cells from 1 cc. of blood in 20 cc. of 0·85 % NaCl. Of this suspension, 0·4 cc. was added to 1·6 cc. of a solution of lysin of such a strength as to give a known dilution of lysin after the mixture. In this way the dilution of lysin

[1] In the paper quoted, the final x in the denominator has been dropped by a printer's error

upon which the calculations are based is that present in the fluid in which the cells are suspended at the beginning of the haemolytic reaction. The lysins were all dissolved in 0·85 % NaCl.

We are here concerned with the extent to which equation (1) describes these time-dilution curves, and shall therefore tabulate experimental and calculated results. In these tables, the dilution of lysin will be replaced by a concentration—the number of milligrams of lysin present in the tube containing 2 cc. of fluid. This is for convenience in calculation only.

In using equation (1) for the calculation of the time-dilution curves, we may simplify it by putting S equal to unity, and ψ equal to zero. The expression then reduces to the well-known expression for a monomolecular reaction

$$\kappa t = \log \frac{c}{c-x}.$$

It cannot be too strongly emphasised, however, that this simplification is permissible only because of the special use to which the expression is to be put: in general the simplification cannot be made.

Concentration mg.	t experimental mins.	t calculated mins.
1. Ammonia and human erythrocytes at 25°		
		$x=15$, $\kappa=0{\cdot}25$
64	0·9	1·0
34	2·1	2·2
23	4·1	4·25
17	9·0	8·5

Concentration mg.	t experimental mins.	t calculated mins.
2. Sodium stearate and human erythrocytes at 25°		
		$x=0{\cdot}32$, $\kappa=0{\cdot}20$
2·0	0·9	0·85
1·0	2·0	1·9
0·5	4·6	5·1
0·4	8·0	8·0
0·3	16·0	15·0

3. Sodium palmitate and human erythrocytes at 25°		
		$x=0{\cdot}25$, $\kappa=0{\cdot}25$
2·0	0·5	0·56
1·0	1·0	1·0
0·5	2·6	2·8
0·4	4·2	4·0
0·3	7·7	7·2
0·285	9·0	8·4

4. Sodium oleate and human erythrocytes at 25°		
		$x=0{\cdot}048$, $\kappa=0{\cdot}166$
0·2	1·5	1·56
0·1	3·6	3·8
0·06	8·0	7·8
0·05	20·0	19·2

5. Sodium taurocholate and human erythrocytes at 25°		
		$x=0{\cdot}33$, $\kappa=0{\cdot}178$
2·0	0·8	0·9
1·0	1·9	2·1
0·5	5·8	6·0
0·4	9·7	9·7

6. Saponin and human erythrocytes at 25°		
		$x=0{\cdot}028$, $\kappa=0{\cdot}303$
0·2	0·4	0·49
0·1	1·0	1·1
0·05	2·7	2·7
0·04	4·0	4·0
0·03	6·2	6·1
0·0285	13·0	12·8

These results are sufficient to show that the degree of exactness with which the times for complete haemolysis can be calculated from equation (1) is considerable. The nature of the lysins, too, is sufficiently varied to show that the expression is one of general applicability. This list might be greatly extended by including the action of each of these lysins on the cells of various

animals; it has been thought unnecessary to do this, for we have shown in the first paper of this series that the relation of the action of saponin on human cells to the action on the cells of any of the mammalia is such that the resistance of the two systems can be expressed by a constant R. Reference to another publication [Ponder, 1926, 2] will show that the appearance of this constant indicates that, if the action of saponin can be described by an expression such as (1), the action on the cells of the sheep, dog, cat, or whatever animal is selected, must be described by an expression of the same type.

We might extend the list by including other lysins. Those given above are the common ones, and the action of the rarer lysins must be left for future investigation.

2. The zone of action.

In a haemolytic system such as one containing red cells and saponin, it is plain that the reaction which results in lysis cannot occur generally throughout the system, but only in the neighbourhood of the cell surfaces. The reaction is therefore discontinuous, and in order to take the idea of discontinuity into account, we use the idea that each cell is surrounded by a zone within which the reaction can be localised, and to which the term "zone of action" is applied.

It is shown in the paper quoted above [Ponder, 1926, 2] that the size of the zone of action may be approximately obtained in a very simple way. We take the most dilute suspension of red cells with which it is possible to work, plot its time-dilution curve, and find $c_{1\infty}$, the concentration of lysin which corresponds to the asymptote. A suspension of a known cell content, some four times greater than that of the very dilute suspension, is then prepared, its time-dilution curve plotted, and $c_{2\infty}$, the concentration of lysin corresponding to the asymptote found.

Then we have $$\frac{c_{2\infty}}{c_{1\infty}} - 1 = \psi \quad \text{.....(2).}$$

But in the nomenclature of another paper [Ponder, 1926, 1], the ratio $c_{2\infty}/c_{1\infty}$ is equal to R, the resistance constant of the second suspension as compared with the first, and this constant has the same value for all values of t. In order to avoid confusion in what follows, we shall call the resistance constant, when it is obtained from two suspensions such as the above for the purpose of working out the dimensions of the zone of action, not R, but R_z. So we have

$$R_z - 1 = \psi,$$

or $$R_z - 1 = v/V \quad \text{.....(3),}$$

where v is the volume of all the zones of action added together, and V the volume of the whole haemolytic system.

Call v' the volume of a single zone of action, and we get

$$\frac{V(R_z - 1)}{N} = v' \quad \text{.....(4),}$$

where N is the number of cells in the haemolytic system which contains the greater number of cells.

If ρ is the radius of the zone of action measured from the surface of the cell outwards (see Fig. 1),

$$\rho = \sqrt[3]{\frac{3V(R_z-1)}{4\pi N}} - r \quad \text{.....(5),}$$

where r is the radius of the cell in the spherical or Goughian form.

The determination of the dimensions of the hypothetical zone of action is therefore an easy matter, for all that has to be done is to determine R_z for a suspension of known cell content and of arbitrary strength as compared with the most dilute suspension with which it is possible to work.

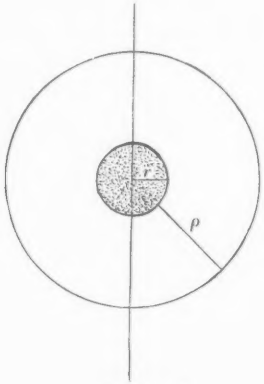

Fig. 1.

Diagram of the zone of action. The cell of radius r is surrounded by the zone, extending outwards for a distance ρ. The figure rotated about the vertical line gives a solid of volume v'. The volume of the sphere representing the cell is v'', and the volume of the spherical zone surrounding it is Z.

Suitable strengths of the suspensions are as follows: (*a*) a suspension containing in 20 cc. of 0·85 % NaCl the thrice washed cells from 1 cc. of blood; (*b*) a suspension containing one-fourth of the cells in suspension (*a*). With a few exceptions, it is impossible to obtain good end-points with suspensions less concentrated than (*b*); in any case, the zones of action appear in such suspensions to be completely separated, which is the essential point.

When two suspensions such as these are used, R_z will always be found, irrespective of the type of cell dealt with, to be less than 2. This is, of course, essential, for if R_z exceeds 2, the zones of action completely overlap, and the limits within which the experimental procedure is possible are overstepped.

We shall illustrate the method by an example.

Lysis of sheep cells by saponin. The strengths of the two suspensions were as in (*a*) and (*b*) above.

For suspension (*a*) the asymptote of the time-dilution curve at 25° was found to correspond to a concentration of lysin of 0·210 mg.

For suspension (*b*) the asymptote corresponded to a lysin concentration of 0·130 mg.

Dividing the one figure by the other we get $R_z = 1{\cdot}63$, and so

$$(R_z - 1) = 0{\cdot}63.$$

This figure is less than 1, as it ought to be. The number of cells in the system was $2.(10^8)$, the volume of the system 2 cc., and the radius of the sheep cell, in the spherical form, 2μ.

Applying equation (5) with these values inserted, we find that the value of ρ works out as $9{\cdot}5\mu$. The zone of action accordingly extends, in the case of this haemolytic system, from the cell surface outwards for a distance of $9{\cdot}5\mu$.

We now proceed to apply this method to the cells of various animals, with a view to determining whether the radius of the zone of action is constant, or whether it varies with the type of cell to be haemolysed.

All the experiments were carried out at 25°. The results are shown in the following table, the types of cell being arranged according to increasing size of the zone of action:

Animal	R_z	$N \times (10^{-8})$	r, μ	ρ, μ
Rabbit	1·07	1·0	2·5	4·5
Man	1·14	1·0	3·0	5·8
Dog	1·18	1·4	2·5	6·0
Cat	1·23	1·2	2·3	7·4
Ox	1·59	1·6	2·3	9·0
Sheep	1·63	2·0	2·0	9·5

A glance at the table will show the remarkable nature of the result, for in the table we have the cells arranged in Ryvosh's series [Ryvosh, 1907; Ponder, 1927]. The order of the cells arranged according to the size of the zone of action is thus the same as the order of the cells arranged according to resistance to saponin. The most resistant type of cell—that of the sheep—has the largest zone of action, the least resistant type—that of the rabbit—the smallest zone of action.

Turning to the idea of the zone of action as a zone within which there exists a concentration gradient of lysin, and regarding its boundary as a point where the slope of this concentration gradient becomes negligible, we see at once the meaning of this important result. In the case of a cell which is very resistant to saponin, such as that of the sheep or ox, a large amount of saponin requires to be used up in order to bring about lysis. As a result, the zone of action is large. If, on the other hand, we have a cell such as that of man or of the rabbit, little saponin needs to be used up to bring about lysis, and the zone of action will be comparatively small. That the size of the zone of action must be related to the resistance of the particular type of cell to the lysin of the system is therefore plain from first principles, and no result other than that of the above table could be expected.

We may, however, go further, and try to find the exact relation between the size of this zone of action and the resistance presented by the particular type of cell. As has been seen in previous papers, the resistance of a particular haemolytic system, compared with that of another haemolytic system

chosen as a standard, may be expressed as the value of a constant R. This constant is obtained by dividing the concentration of lysin which corresponds to the asymptote of the time-dilution curve at 25° (or any other convenient temperature), for a haemolytic system containing saponin and the particular type of cell whose resistance is to be determined, by the concentration of lysin corresponding to the asymptote of the time-dilution curve at 25° for a haemolytic system containing the cells to be used as a standard, together with saponin. Usually it is convenient to use human cells as the standard type. For the detail of this method a previous paper [Ponder, 1926, 1] should be consulted.

In using this method of comparing resistance of cells, it is to be remembered that R refers to an entire haemolytic system. If we wish to use values of R to show the resistance of the individual cell to the lysin—or, what is the same thing, to show the amount of lysin combining with the individual cell—we must be very careful that the haemolytic systems from which the values of R are obtained are in every respect comparable. They must be comparable as regards the state of the zones of action in the system; it is no use comparing two systems in one of which the zones overlap and in the other of which they are separate. Further, if R is to indicate the amount of lysin used up by the single cell, the systems must have the same cell content. We must therefore determine values of R for systems in which the zones of action are all separate, and in which the chance of a cell entering the zone of action of another cell is so small as to approach zero. Having done this, the systems have to be reduced to the same red cell content. This point deserves the most careful consideration. A value of R determined in this way may be properly compared with figures for the dimensions of the zone of action.

With these values of R/N we shall tabulate values of the volume of the zone of action. Call this volume Z; it is obtained from $(v' - v'')$, where v' is given by equation (4), and where v'' is the volume of the cell supposed to occupy the centre of the zone:

Animal	$R/N \times (10^8)$	v', μ^3	v'', μ^3	Z, μ^3
Rabbit	0·46	1400	65	1335
Man	0·88	2800	110	2690
Dog	0·71	2540	65	2485
Cat	1·4	3830	51	3779
Ox	2·5	7275	51	7224
Sheep	2·15	6300	33	6267

If the values of R/N be plotted against the corresponding values of Z, a fairly good straight line will be obtained. This line is shown in Fig. 2. The points lie along the line in a very satisfactory manner considering the amount of calculation necessary in their derivation.

The equation of this line is

$$Z = \frac{3.(10^{11}).R}{N} \quad \text{.....(6),}$$

in which R refers to suspensions in which the zones of action are completely separated, and in which Z is given in μ^3.

We may compare the experimental results with those given by this expression, and summarise the results in the following table:

Animal	Z, exper.	Z, calc.
Rabbit	1335	1380
Man	2690	2640
Dog	2485	2130
Cat	3779	4200
Ox	7224	7500
Sheep	6267	6750

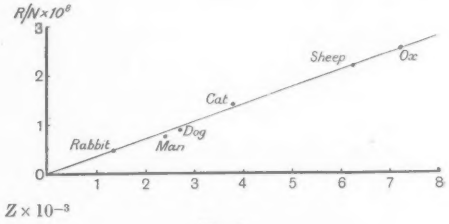

Fig. 2.

Bearing in mind that Z is in μ^3, the correspondence is excellent.

The volume of the zone of action is thus proportional to the amount of lysin used up by the cell in the haemolytic reaction. This result, it will be observed, is by hypothesis the only possible one, for we have defined the zone of action as containing the lysin which reacts with the cell. While, however, the result could have been obtained from the original conditions as a deduction, we have here obtained it in a somewhat different way, by the consideration of Ryvosh's series, and have thus linked up this series with the fundamental equation (equation (1)), with the conception of the zone of action, and with the resistance constants for the different types of cell. The fact that so many different methods of attack yield the same result is the strongest evidence of the correctness of all the underlying assumptions, and of the applicability of the fundamental equation to the kinetics of a simple haemolytic system in which saponin is the lysin.

It will be appreciated that there is much room for the extension of these methods to other haemolytic systems. These extensions we propose to deal with later.

Finally, it is instructive to represent the conception of the zone of action, regarded as a concentration gradient, in a graphical form. This is done in Fig. 3. The cell, of radius r, is represented by a thick line. At its surface the concentration of lysin is taken to be zero, and in the concentration gradient of radius ρ this concentration is supposed to rise to a value given by R/N as measured in equation (6). Between the values of R/N and zero the concentration falls according to some curve which we cannot at present determine, so we represent it merely by a straight line. In fact, it is probably a curve of exponential character. These lines are shown in the diagrams, one of which refers to the cell of the rabbit, while the other refers to the cell of the sheep.

The fall in the case of the sheep cell is very marked, and the concentration gradient very steep, as we should expect from the great resistance of this type of cell to saponin; in the case of the rabbit cell, the concentration gradient is much less steep, corresponding to the relatively low resistance of this type of cell.

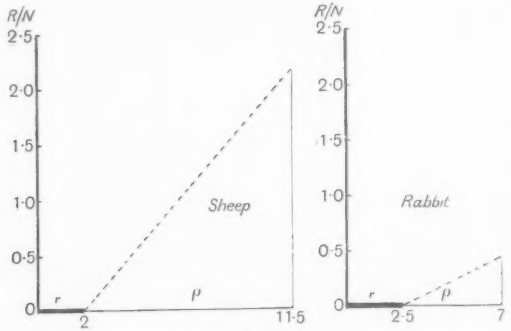

Fig. 3.

Although these figures are drawn to scale, it will be understood that they represent only in a very approximate way the conditions present, and that they are to serve no purpose except to give a general idea of the state of affairs at the cell surfaces.

SUMMARY.

1. A series of tables comparing experimental and calculated results for different haemolytic systems is given, together with the expression used for obtaining the calculated results.

2. A method for determining the dimensions of the zone of action round the cells of a haemolytic system is described. The radii of the zones are determined for the cells of different animals, and it is shown that the cells, arranged in order of increasing size of zone of action, fall into Ryvosh's series.

3. It is shown that the volume of the zone of action is proportional to the resistance of the cell to the lysin, a relation which follows from theoretical considerations.

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REFERENCES.

Ponder (1926, 1). *Proc. Roy. Soc. Lond.* B, **99**, 461.
—— (1926, 2). *Proc. Roy. Soc. Lond.* B, **100**, 199.
—— (1927). *Biochem. J.* **21**, 56.
Ryvosh (1907). *Pflüger's Arch.* **116**, 229.

XIX. THE RELATION OF CHOLESTEROL TO VITAMIN D.

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In continuation of our work on the molecular groupings essential for the successful activation of sterols by ultraviolet light [Rosenheim and Webster, 1926] we had the privilege of collaborating with Prof. Windaus of Göttingen in the examination of various isomers of cholesterol, recently prepared by him, and of other sterols. The details of this work which gave entirely negative results [see also Hess and Windaus, 1926] we hope to publish shortly in conjunction with Prof. Windaus.

Our failure to find any derivatives of sterols which were either active by themselves or could be rendered antirachitic by irradiation, led us to a reconsideration of the problem. The following facts emerge from previous work.

1. At least 99·9 % cholesterol is recovered unchanged when the active substance is separated from cholesterol, irradiated in a nitrogen atmosphere, by precipitation with digitonin. Further, no appreciable difference in activity is observed when the time of irradiation is varied from only a few minutes to many hours. Apparently only a small amount of cholesterol can be converted into the active condition.

2. Changes produced in the cholesterol molecule by oxidation of the hydroxyl group, or replacement of this group by chlorine, or its complete removal, deprive the derivatives so formed (cholestenone, cholesteryl chloride, cholestene) of the power to be rendered antirachitic by ultra-violet light. The presence of the OH group appears therefore essential, but the fact that the esters of cholesterol (acetate, palmitate) can be activated is contrary to this conclusion, unless the assumption is made that the esters are slightly hydrolysed during irradiation.

3. Phytosterols prepared from a sample of corn oil, which had been kept many years, cannot be activated [Steenbock and Black, 1925]. Further, another phytosterol, stigmasterol, prepared by Windaus and Hauth [1906] by way of the tetrabromide, has been found by Windaus to be incapable of being activated, a fact which we were able to confirm.

4. Allo-cholesterol, a new isomer of cholesterol recently discovered by Prof. Windaus, cannot be activated although it easily changes into cholesterol. We have recently been informed by Prof. Windaus that the actual specimen of allo-cholesterol examined by us contained some free cholesterol.

Arising out of these considerations, it seemed to us essential to investigate the question, how far the purity of the cholesterol employed is related to its capacity for activation by ultra-violet light. Although we ourselves and others had used specimens of cholesterol which had been purified with special care by the usual methods (saponification and recrystallisation, etc.) we decided to purify it still further by a chemical method. For this purpose a many times recrystallised specimen of cholesterol (M.P. 148–9°) was converted into the dibromide, and the latter again reduced to cholesterol by means of sodium amalgam in the presence of acetic acid. The melting point of the purified substance remained unchanged (148–9°) and it gave the usual colour reactions with great intensity.

This product was irradiated for 1 hour and tested biologically by the methods previously used [Rosenheim and Webster, 1926].

Details of the animal experiments are given in the following table:

Rat	Daily dose mg.	Inorganic blood phosphate mg. %	X ray result
1	2	2·3	Rickets
2	4	2·4	,,
3	8	2·4	,,
4	Control	2·2	,,
5	,,	2·3	,,

The result of this series of experiments is striking, especially when we consider that the original cholesterol preparation, when irradiated, prevented rickets in rats even when administered in such small doses as 0·5 mg. *per diem* [Rosenheim and Webster, 1925]. Purification by way of the dibromide completely deprived this cholesterol, which would previously have been considered as "chemically pure," of its power to become antirachitic by irradiation with ultra-violet light.

This observation throws new light on the photo-chemical formation of vitamin D. It is evident that the precursor of vitamin D is not cholesterol itself, but a substance which is associated with and follows "chemically pure" cholesterol in all its stages of purification by the usual methods (esterification, saponification, recrystallisation).

The possibility that the absorption spectrum of cholesterol may be due to a small amount of an impurity had indeed already been suggested by Schlutz and Morse [1925] in their careful study of the absorption spectra of cholesterol. The precursor of vitamin D[1] need not necessarily be an extraneous impurity of ordinary cholesterol. When we consider the ease with which sterols form stable complex additive compounds such as the "phytosterol" of calabar beans [Windaus and Hauth, 1906] and the complex of β-cholestanol with pseudocoprosterol [Windaus and Uibrig, 1915], the possibility must be kept

[1] In our first communication (*J. Soc. Chem. Ind.* **45**, 932) we proposed the name "Vitasterol" for the precursor of vitamin D, but as this term might lead to confusion we have adopted the more expressive name "Provitamin," suggested by Prof. Windaus, for the parent substance of vitamin D.

in mind that the provitamin may be a substance allied to cholesterol in character. The amount present in ordinary "pure" cholesterol may be judged from the yield of active substance obtained by us [Rosenheim and Webster, 1926] in the digitonin experiment, to be of the order of $\frac{1}{10}$ %. Preliminary experiments seem to justify the statement that provitamin D is precipitated by digitonin and that its separation from cholesterol may be possible by fractional precipitation or extraction of the digitonin complex.

It is obvious that the nature of this unidentified substance, its relation to cholesterol, and its separation from it can now be investigated by experimental means.

In view of the spectrographic work of Hess and Weinstock [1925], and of Schlutz and Morse [1925] it is suggestive that, according to information kindly supplied by Prof. Windaus, a specimen of cholesterol, prepared by him at our suggestion by way of the dibromide, no longer showed the absorption spectrum in the ultra-violet region characteristic of ordinary "pure" cholesterol. This specimen had been converted into the dibromide, which was recrystallised twice and then reduced with zinc dust in the presence of acetic acid, the whole series of operations being repeated.

It is interesting to note that Heilbron, Kamm and Morton [1926] as the result of an analysis of the ultra-violet absorption spectra of cholesterol make the suggestion that cholesterol may not be the precursor of vitamin D, but that the activation of ordinary cholesterol may be due to the presence in it of traces of an unknown substance[1].

REFERENCES.

Heilbron, Kamm and Morton (1926). *J. Soc. Chem. Ind.* **45**, 932; see *Biochem. J.* (1927), **21**, 78.
Hess and Weinstock (1925). *J. Biol. Chem.* **64**, 193.
Hess and Windaus (1926). *Proc. Soc. Exp. Biol. Med.* **24**, 171.
Rosenheim and Webster (1925). *Lancet*, i, 1025.
—— —— (1926). *Biochem. J.* **20**, 537.
Schlutz and Morse (1925). *Amer. J. Dis. Child.* **30**, 199.
Steenbock and Black (1925). *J. Biol. Chem.* **64**, 263.
Windaus and Hauth (1906). *Ber. deutsch. chem. Ges.* **39**, 4378.
Windaus and Uibrig (1915). *Ber. deutsch. chem. Ges.* **48**, 857.

[1] Our biological test of two preparations of these authors is referred to in their paper in this number of the Journal.

[Addendum: Jan. 31st, 1927.] Since the above communication went to Press, it has been found that ergosterol, or a sterol of similar constitution, is the parent substance of vitamin D. A preliminary communication by ourselves on this work is in the Press (see *Lancet*, Feb. 4th, 1927) and a similar one will be made in Germany by Prof. Windaus.

XX. STUDIES ON THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA. PART I.

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(Report to the Medical Research Council.)

(Received November 27th, 1926.)

During the past 20 years a very large number of observations have been published on the total cholesterol content of blood or of serum from pathological cases. As a result, the blood picture is described, in any one case, as a hypercholesterolaemia or a hypocholesterolaemia, in comparison to a so-called normal value of total cholesterol in human blood. It is, however, to be noted that relatively few determinations have been made on normal subjects, and that values of the normal, obtained from these sparse data—not too concordant in the different researches—have been quoted from author to author.

It is generally agreed that in certain forms of nephritis, irregularly in diabetes mellitus and also in the later months of pregnancy, a hypercholesterolaemia is found, and that in infective conditions a hypocholesterolaemia occurs. As regards other diseases it is impossible in the present state of our knowledge of the physiological limits of cholesterol content of the blood to decide if there is any constant alteration in the level of this value. Denis [1917] considers that at present the total cholesterol content of the blood is practically of no diagnostic significance.

Though much work has been done on the total cholesterol content of blood or serum, comparatively little attention has been directed to the relative amounts of so-called free or non-esterified cholesterol and cholesterol in ester form. This unfortunate position is to some extent explained by the historical development of our knowledge of this subject. Fat, cholesterol and allied substances were obtained together by the extraction of tissues and fluids of the body by means of ether and other solvents, and in early days no effective methods were available for differentiating, quantitatively at any rate, between free and ester cholesterol. Consequently cholesterol came to be regarded as intimately connected with fat metabolism.

This idea has persisted throughout the history of the subject and has been upheld largely by the French observers. Terroine [1919] considers that there is a constant lipaemic coefficient, *i.e.* a constant ratio of total cholesterol to total fatty acid in individual animals. Chauffard and his school [1920]

consider that "normally there exists a tendency in the organism to preserve the equilibrium between the different elements of the lipaemia, fats, cholesterol and lecithin, and that it is probable that there exists a regulator mechanism which controls at the same time the amounts of the fats and lipins and the relative proportion of these different elements amongst themselves."

Further, Chauffard and his collaborators consider that cholesterol metabolism depends on three factors: (1) alimentation, (2) the glands of internal secretion, particularly the suprarenal cortex, (3) the liver. However, this view does not appear to lay sufficient stress on the fact that sterols are integral constituents of all living cells.

That so much attention has been concentrated on the total cholesterol of blood would seem to imply that the existence of a portion of this total in the form of ester is a purely fortuitous condition of no particular importance. However, a consideration of the different distribution of free and ester cholesterol throughout the body, noticeably in the brain, red blood corpuscles and in the bile would suggest that such an assumption is untenable.

In recent years cholesterol balance experiments by Gardner and Fox [1921, 1, 2; 1925] and by Thannhauser [1923] have shown that in the normal human adult there is always a small negative balance, that is, an excess of output over intake, which indicates that though the food may be a source of cholesterol, nevertheless there must be some synthesis in the body. That such synthesis does take place has been shown by the study of the relative growth and cholesterol content of infants' brain and by experiments with dogs by Beumer and Lehmann [1923], and with rats by Channon [1925] and by Randles and Knudson [1925].

Beumer [1923] goes so far as to advance the view that cholesterol is not to be considered as a food in any sense of the term but that in metabolism it plays a part more comparable with the rôle of the purines, in the sense that the cholesterol excreted by the organism is the true residuum, unrequired by the body, of the metabolic processes and is derived like uric acid from endogenous and exogenous sources. Whatever may be the truth of this conception it does not appear to us to modify the views previously maintained by Gardner in former papers of the nature of the cholesterol cycle.

In considering these different views it does appear to be certain that at the present time we have not the data on which to base a sound theory, nor data enough to criticise these views.

It appeared to us therefore to be of great immediate importance to study the free and ester cholesterol content of the plasma of normal individuals, and to examine the limits of its physiological variations and the conditions that cause such variations, also, if possible, to get evidence of the actual state of existence of the cholesterol in the blood. A general survey of the results hitherto obtained seems to justify the view that changes in the total cholesterol content of blood, or of the ratio of the free to ester forms, take place mainly in the plasma, and that the content of corpuscles is relatively if not

quite constant, and consists mainly of free cholesterol. In this paper we give the results of a number of analyses of the plasma of normal subjects.

Technique.

The methods in use for estimating cholesterol and cholesterol esters in various tissues were critically dealt with by Gardner and Williams [1921] and by Gardner and Fox [1921, 1, 1924], and our experience has not led to the modification of the conclusions expressed in these papers. As the analyses of blood and plasma present rather more difficulties than those of other tissues, when both free and ester cholesterol are to be estimated, and rather conflicting statements have been made in recent years, notably by Bloor and Knudson [1916, 1917] and by Richter-Quittner [1920], we think it may be useful to give a more detailed description of our methods than has hitherto been published.

In our experience two methods of extraction are practically equally effective, viz., the method of Fex [1920] and the alcohol-ether method advocated by Bloor. In the case of whole blood about 10 cc. of blood are withdrawn from a vein and treated at once. If the plasma is to be analysed, it is better to draw a sufficient quantity of blood to yield at least 10 cc. of plasma into a tube containing a small quantity of solid potassium oxalate (0·2 %).

The blood is at once centrifuged and the plasma separated and weighed. A convenient quantity is about 10 to 15 g. It has been objected by Richter-Quittner that sodium oxalate and sodium fluoride tend to injure the corpuscles and should not be used. He recommends sodium citrate. In our experience, however, if the blood is centrifuged soon after drawing, the plasma shows no trace of haemolysis.

In Fex's procedure the fluid—whole blood or plasma—is diluted with about 10 to 15 times its volume of 2 % caustic soda, and after standing for several hours is heated for ½ to 1 hour on the water-bath. After cooling the fluid is thoroughly extracted in a separator by ether. This is best done as recommended by Fex; the alkaline fluid is placed in the separator with about half its bulk of ether, and thoroughly shaken at intervals during the day. After standing over night the ethereal solution is separated, replaced by a new quantity of ether and the alkaline fluid and the ether again shaken at intervals during the day. This process is repeated four or more times, until the ether no longer takes up anything from the alkaline fluid. The clear ethereal solution is then distilled to dryness on the water-bath, and the residue taken up in alcohol.

In the other process the weighed blood or plasma is run in a thin stream into about 250 cc. of a mixture of three parts alcohol to one part ether, which is kept gently rotating. The fluid is then heated to boiling on the water-bath. After cooling, the liquid is filtered through a small filter and the proteins very thoroughly washed by decantation with at first the alcohol-ether mixture and then with ether, any protein on the filter being returned at each washing to the flask: the protein is then washed with boiling alcohol and finally again

with ether. The ether-alcohol mixture is then distilled to dryness on the water-bath. The dry residue is boiled out thoroughly with small successive quantities of alcohol and the alcoholic extracts passed through a small filter. In this case it is necessary to filter, as the original ether-alcohol solution contains traces of protein and some of the plasma salts, which are not dissolved by the alcohol. At this stage it is advisable to wash the residue on the filter with ether and put the ether extract into the flask containing the residue insoluble in alcohol. The subsequent hydrolysis of the esters of cholesterol may then be done in this flask. Esters of cholesterol are not very soluble in alcohol, and this procedure obviates any loss of ester.

The free and ester cholesterol in the alcoholic solution, prepared by either method, are estimated by means of digitonin. Our procedure is as follows. The hot alcoholic solution is mixed with an excess of an alcoholic solution of digitonin in a small beaker and brought to boiling point. After cooling, water is added drop by drop until the alcohol is sufficiently diluted for the cholesterol digitonide to begin to deposit. After standing over night the fluid is carefully evaporated to dryness on the top of a water-bath. The contents of the beaker are then treated with three successive portions of 15 to 20 cc. of dry ether and these ether washings filtered through a weighed Gooch crucible and carefully collected. In this way the cholesterol esters along with any resinous matter are obtained in ethereal solution. The digitonide of the free cholesterol is then treated with boiling water to dissolve excess of digitonin, and brought on to the Gooch crucible and the washing completed with hot water. The filtrate should be water-clear, otherwise refiltered until it is. This washing process is complete when the washings, on vigorous shaking, show no trace of frothing. The Gooch crucible and its contents are then dried first at 100° and finally at 110° to constant weight.

The ethereal solution of the esters, etc., is poured into a flask provided with a reflux condenser (for this purpose the flask which held the original ether or ether-alcohol extract is used) and a large excess of an alcoholic solution of sodium ethoxide added, and after standing over night is boiled for at least 8 hours. After cooling, the alcoholic solution is largely diluted with ether and shaken in a separator with water. The aqueous solution so obtained is extracted three times with ether and the ether layer added to the main ether solution. The alkaline fluid is then again extracted in a second separator with a comparatively large quantity of ether, and the alkaline fluid then run off. The main ethereal solution formed by hydrolysis of the esters is then repeatedly shaken with successive portions of water to remove any traces of soap, and the aqueous washings shaken with the ether left in the second separator to avoid any risk of loss of cholesterol. The whole of the ether solution is then distilled to dryness on the water-bath, the residue taken up in alcohol and the cholesterol precipitated with digitonin and treated as described above.

This procedure presents advantages over the original method of Windaus [1910] which is used by most observers, especially in the case of fluids such

as blood which contain only small quantities of cholesterol or cholesterol esters, since any loss of cholesterol digitonide through slight solubility in alcohol is obviated. Such loss in the Windaus process is difficult to allow for, particularly when the quantity of cholesterol digitonide is small. The error due to this solubility is obviously the more serious the smaller the quantity to be weighed.

Results.

In Table I we give the results of a few duplicate analyses, both by Fex's and the alcohol-ether methods, which give an idea of the experimental errors to which the method of analysis is liable when quantities of the order mentioned are dealt with. When the complexity of the operation involved in the analyses is taken into account, we consider these results satisfactory.

Table I.

Method of extraction	Wt. of whole blood or plasma in g.	Wt. of cholesterol digitonide (g.) from (*a*) Free cholesterol	(*b*) Ester cholesterol	Cholesterol (g.) per 100 g. of whole blood or plasma: Free	Ester	Total	Remarks
Fex ...	10·3846	0·0378	0·0244	0·0885	0·0571	0·1456	Whole blood from normal male
	10·3802	0·0384	0·0244	0·0899	0·0576	0·1475	
Alcohol-ether	8·3461	0·0402	0·0246	0·1171	0·0716	0·1887	Whole blood taken from a case of mitral stenosis
Fex ...	10·4383	0·0462	0·0320	0·1076	0·0745	0·1821	
	10·3287	0·0453	0·0308	0·1069	0·0727	0·1796	
Alcohol-ether	15·531	0·0414	0·0706	0·0648	0·1105	0·1753	Plasma from a case of myocarditis
	15·531	0·0423	0·0770	0·0662	0·1205	0·1867	
Fex ...	15·531	0·0402	0·0742	0·0629	0·1161	0·1790	
	15·531	0·0389	0·0705	0·0609	0·1104	0·1713	

Though these two methods of extraction give results in practical agreement, and in our experience are the best available when both free and ester cholesterol have to be estimated, nevertheless they are not perfect. Small quantities of cholesterol or cholesterol esters remain persistently in the proteins as the following experiments, selected from many, show.

Alcohol-ether method.

1. The protein collected after extraction from six cases, representing 72·7 g. of plasma, was dissolved in 2 % caustic soda and repeatedly extracted with ether in the manner described above. The ether extract gave 0·0009 g. of compound from free cholesterol, and 0·0011 g. from ester cholesterol. The extracted alkaline protein was then evaporated to dryness and boiled for 4 to 5 hours with glacial acetic acid containing hydrochloric acid. The acid liquor was then neutralised and thoroughly extracted with ether. The ether extract was then hydrolysed by sodium ethoxide to saponify any cholesterol acetate, and again extracted. The ether extract yielded 0·0026 g. of cholesterol digitonide. These figures correspond to 0·00031 % of free cholesterol, and 0·00038 % of ester cholesterol left in the protein and 0·0009 % freed by acid hydrolysis.

2. 7·95 g. of dried protein from 114·3 g. of extracted plasma of nine cases were treated in a similar manner. 0·0045 g. of compound from free cholesterol, 0·0061 g. from ester cholesterol and a trace after acid hydrolysis were obtained. These figures represent 0·00096 % free cholesterol and 0·0013 % ester cholesterol left in the original plasma.

Fex method.

3. 50·15 g. extracted plasma from five cases were very thoroughly re-extracted with ether, and the ethereal extract hydrolysed by sodium ethoxide and again extracted. From this 0·0058 g. of compound was obtained, equivalent to 0·0028 % of cholesterol (free and combined). After acid hydrolysis, as above, an extract was obtained which yielded 0·0012 g. compound equivalent to 0·0006 % cholesterol. The average error in these cases due to inefficient extraction was therefore 0·0034 g. per 100 g.

4. 93·1 g. of extracted plasma from seven cases were very thoroughly re-extracted with ether and the extract hydrolysed. From the extract was obtained 0·0011 g. compound, equivalent to 0·00029 g. cholesterol (free and ester) per 100 g. of original plasma. After acid hydrolysis it yielded 0·0054 g. compound, equivalent to 0·0014 g. cholesterol per 100 g. plasma.

These average errors due to inefficient extraction are obviously negligible, unless perchance they happened to be all concentrated in one sample, which, owing to the care taken, was very improbable, and are probably well within the variations caused by changes in the plasma concentration during the day.

Cholesterol content of plasma of normal individuals.

The subjects of our experiments were drawn chiefly from students in our classes at the London School of Medicine for Women and at St George's Hospital, from nurses, laboratory attendants and colleagues. They were all in normal health, as far as could be judged clinically. The samples of blood were drawn from an arm vein, and were collected in a few cases from fasting patients, *i.e.* before the first meal of the day, but in most cases about 4 to 5 hours after breakfast or a light lunch. In Table II we give essential details and results of our experiments.

Discussion of results.

Analyses of the plasma of normal human subjects have been published in recent years by Bloor and Knudson and by Richter-Quittner, who came to rather opposite conclusions. Bloor and Knudson [1917] examined 17 men and 9 women. The total cholesterol in plasma was determined by the colorimetric method of Bloor, and the ester cholesterol by the same method after elimination of free cholesterol by means of digitonin. The colorimetric method of Bloor [1916] is well known to give higher results than the digitonin method, and their average results are, as might be expected, higher than our own. They conclude that in normal human plasma there is a constant relation

Table II.

Women

No. of case	Age of subject	Time after last meal Hours	Nature of meal	Catamenia	Anticoagulant used	Appearance of plasma	Method of extraction	Wt. of plasma in g.	Wt. of digitonide (g.) from Free cholesterol	Wt. of digitonide (g.) from Ester cholesterol	Amount of cholesterol (g.) in 100 g. of plasma: Free	Ester	Total	Ratio free : ester	
1	20	4	Breakfast	Between periods	K oxalate	Clear, no haemolysis	Fex	11·1515	Lost	0·0457	—	0·0996	—	—	
2	19	4	,,	,,	,,	,,	,,	10·1138	0·0137	0·0544	0·0329	0·1308	0·1637	1 : 3·97	
3	19	4	,,	,,	,,	,,	,,	13·2724	0·0112	0·0314	0·0205	0·0575	0·0780	1 : 2·8	
4	21	5	,,	,,	,,	,,	,,	13·1465	0·0223	0·0738	0·0412	0·1365	0·1777	1 : 3·32	1
5	20	3	,,	,,	,,	Yellow, sl. opalescence	,,	18·4537	0·0323	0·0489	0·0426	0·0644	0·1070	1 : 1·52	
6	20	4	,,	,,	,,	,,	,,	14·2678	0·0210	0·0319	0·0358	0·0544	0·0902	1 : 1·42	
7	19	5½	,,	,,	,,	,,	,,	12·6901	0·0294	0·0509	0·0563	0·0975	0·1538	1 : 1·73	
8	18	4	,,	,,	,,	Clear, v. sl. haemolysis	,,	7·4765	0·0210	0·0303	0·0600	0·0867	0·1467	1 : 1·44	
9	25	4	,,	,,	,,	Opalesc., sl. haemolysis	,,	9·4117	0·0231	0·0283	0·0597	0·0920	0·1517	1 : 1·54	
10	19	4½	,,	,,	,,	Clear, sl. haemolysis	,,	8·464	0·0215	0·0208	0·0628	0·0597	0·1215	1 : 0·97	2
11	24	4½	,,	Just before period	,,	Opalesc., sl. haemolysis	,,	9·5207	0·0262	0·0485	0·0599	0·1108	0·1707	1 : 1·85	
12	17	4½	Light breakfast	Between periods	,,	Clear, v. sl. haemolysis	,,	6·6325	0·0181	0·0292	0·0663	0·1070	0·1733	1 : 1·61	
13	24	4	Light lunch	,,	Paraffined tube	Clear, no haemolysis	Alcohol-ether	10·9658	0·0304	0·0631	0·0675	0·1402	0·2077	1 : 2·08	
14	23	1½	Good lunch	Sl. amenorrhoea	K oxalate	Yellow, clear	,,	10·5254	0·0328	0·0393	0·0758	0·0908	0·1666	1 : 1·20	
15	22	Immediately	Lunch	Between periods	,,	Brownish, clear, no haemolysis	,,	7·5215	0·0172	0·0313	0·0556	0·1012	0·1568	1 : 1·82	
16	20	4½	Light breakfast	Period now on	,,	Clear, v. sl. haemolysis	,,	12·3943	0·0288	0·0538	0·0565	0·1055	0·1620	1 : 1·90	
17	19	6	Breakfast	Sl. amenorrhoea	,,	Clear, no haemolysis	,,	13·3648	0·0519	0·0727	0·0944	0·1322	0·2266	1 : 1·40	
18	18	5½	,,	Between periods	,,	Sl. opalesc., no haemol.	,,	12·1697	0·0356	0·0500	0·0713	0·1001	0·1714	1 : 1·40	
19	20	5½	,,	Sl. amenorrhoea	,,	Clear, no haemolysis	,,	14·2066	0·0252	0·0477	0·0431	0·0816	0·1247	1 : 1·89	3
20	19	4½	,,	Between periods	,,	Clear, sl. haemolysis	,,	12·1956	0·0243	0·0690	0·0458	0·1302	0·1760	1 : 2·84	
21	18	4½	Light breakfast	,,	,,	,,	,,	7·4523	0·0106	0·0281	0·0346	0·0917	0·1263	1 : 2·65	
											Mean 0·0541	0·0986	0·1528		

Men

22	24	3½	Breakfast		K oxalate	Sl. haemolysis	Fex	10·5007	0·0239	0·0602	0·0553	0·1394	0·1947	1 : 2·50
23	24	4½	,,		,,	,,	,,	5·7234	0·0029	0·0217	0·0123	0·0922	0·1035	1 : 7·50
24	22	4	,,	—	,,	Yellow, clear, no haem.	,,	11·5183	0·0315	0 0258	0·0665	0·0546	0·1211	1 : 0·82 [4]
25	23	3½	,,	—	,,	Opalescent, no haem.	,,	12·0843	0·0052	0·0490	0·0105	0·0986	0·1091	1 : 9·40
26	23	3½	,,	—	,,	Clear, sl. haemolysis	,,	10·3250	0·0021	0·0444	0·0049	0·1045	0·1094	1 : 21·3
27	58	Fasting	—	—	,,	Yellow, no haemolysis	,,	9·1213	0·0193	0·0526	0·0514	0·1386	0·1900	1 : 2·69
28	58	2¼	Heavy lunch	—	,,	Opalescent, no haem.	,,	9·8053	0·0163	0·0488	0·0404	0·1210	0·1614	1 : 2·99
29	33	Fasting	—	—	,,	Clear, no haemolysis	,,	10·4763	0·0176	0·0492	0·0418	0·1142	0·1560	1 : 2·73
30	33	2½	Heavy lunch	—	,,	Very milky, green, opalescent	,,	10·4885	0·0162	0·0349	0·0375	0·0809	0·1184	1 : 2·15
31	20–24	4	Breakfast	—	,,	Clear, no haemolysis	,,	21·3406	0·0350	0·1149	0·0418	0·1373	0·1791	1 : 3·29[5]
32	20–58	4	,,	—	,,	,,	,,	10·2090	0·0303	0·0510	0·0722	0·1214	0·1936	1 : 1·68[6]
33	19	5	,,	—	,,	,,	,,	10·2368	0·0187	0·0434	0·0444	0·1033	0·1477	1 : 2·33[7]
34	35	4	Light lunch	—	Paraffin lined tube	,,	Alcohol-ether	10·6456	0·0310	0·0598	0·0754	0·1454	0·2208	1 : 1·93
35	22	4	,,	—	,,	Yellow, clear, no haem.	,,	9·0780	0·0306	0·0526	0·0819	0·1409	0·2228	1 : 1·72[8]
36	58	16	Dinner	—	K oxalate	Clear, v. sl. haemolysis	,,	11·5673	0·0387	0·0546	0·0815	0·1150	0·1965	1 : 1·41
37	23	5′	Light lunch	—	Heparin	Clear brown, no haem.	,,	9·4307	0·0210	0·0249	0·0541	0·0642	0·1183	1 : 1·19
38	33	Fasting	—	—	K oxalate	Clear, no haemolysis	,,	16·3950	0·0405	0·0958	0·0601	0·1420	0·2026	1 : 2·36
39	33	3	Heavy lunch, ample fat	—	,,	Opalescent, no haem.	,,	14·5099	0·0365	0·0787	0·0612	0·1319	0·1931	1 : 2·16
40	23	4	Light lunch	—	Heparin	Clear, sl. haemolysis	,,	11·6176	0·0148	0·0332	0·0310	0·0695	0·1005	1 : 2·24[9]
41	22	2½	Moderate lunch	—	K citrate	Opalescent, sl. haem.	,,	12·1277	0·0263	0·0532	0·0527	0·1066	0·1593	1 : 2·02
42	21	2½	Light lunch	—	,,	Clear, sl. haemolysis	,,	12·9603	0·0331	0·0379	0·0609	0·0711	0·1320	1 : 1·17
43	21	3	Very light lunch, chocolate	—	,,	Sl. opalesc., sl. haem.	,,	12·7706	0·0294	0·0588	0·0560	0·1119	0·1679	1 : 2·00
										Mean	0·0504	0·1193	0·1694	

[1] The extracted alkaline plasma from these 7 cases, on re-extraction and subsequent hydrolysis, gave 0·0011 g. compound equivalent to 0·00029 g. % plasma. After acid hydrolysis gave 0·0054 g. compound equivalent to 0·0014 g. % plasma.

[2] Total alkaline plasma from these 5 cases, on thorough re-extraction, yielded 0·0365 g. compound equivalent to 0·0203 g. %. After acid hydrolysis gave 0·0448 g. compound equivalent to 0·0027 g. %.

[3] Precipitated protein from these 6 cases dissolved in 2 % NaOH and thoroughly extracted with ether gave 0·0009 g. compound from free cholesterol and 0·0011 g. compound from ester cholesterol equivalent to 0·00031 g. free cholesterol per 100 g. plasma and 0·00038 g. ester cholesterol per 100 g. plasma. After acid hydrolysis gave 0·0026 g. compound equivalent to 0·0009 g. per 100 g. plasma.

[4] Total alkaline plasma of these 5 cases, after re-extraction and subsequent hydrolysis, gave 0·0058 g. compound equivalent to 0·0028 g. per 100 g. plasma. After acid hydrolysis gave 0·0012 g. compound equivalent to 0·00058 g. per 100 g. plasma.

[5] Mixed sample from 12 subjects.

[6] Mixed sample of about equal volumes from 6 subjects.

[7] On re-extraction, no free cholesterol but 0·0028 g. compound from ester cholesterol found equivalent to 0·0067 %. After acid hydrolysis 0·0024 g. compound equivalent to 0·0058 %.

[8] The protein dissolved in 2 % NaOH and thoroughly extracted. No free cholesterol but 0·0018 g. compound from ester cholesterol equivalent to 0·0048 % plasma.

[9] Arm somewhat cyanosed on venipuncture.

between free cholesterol and ester cholesterol, the ester cholesterol being 58 % of the total, with a variation from the average in individuals of about 15 % of the average; and further that there is no notable difference in their values between men and women. This constant relation between free and bound cholesterol they consider gives further support to the assumption that cholesterol takes an active part in fat metabolism, and also indicates that for cholesterol esters, as for other "lipoids," there is an efficient regulation. We scarcely think these general conclusions are warranted by their figures, still less by our own. Bloor and Knudson find that the ratio of free to ester cholesterol is on an average 1 : 1·35 in normal men, with individual limits of variation of 1 : 0·84 to 1 : 2·14, and in normal women an average of 1 : 1·49 with limits of 1 : 1·04 to 1 : 2·34. As will be seen from Table II our own average ratios are for men 1 : 2·36, with limits of 1 : 0·82 to 1 : 21·3, or eliminating three cases in which the cholesterol was nearly all in the form of ester, 1 : 2·36 with limits of 1 : 0·82 to 1 : 3·29; and in women an average of 1 : 1·93 with variations of 1 : 0·97 to 1 : 3·97.

On the other hand, Richter-Quittner [1920] on, we think, insufficient normal data, concluded that blood corpuscles contain exclusively free cholesterol and no ester, whereas the *plasma contains exclusively ester and no free cholesterol*. He also draws a distinction between alimentary hypercholesterolaemia and pathological hypercholesterolaemia, but this subject we hope to deal with in another communication.

Richter-Quittner appears to attribute the varying results of other observers to injury to corpuscles in obtaining the plasma, by such causes as too vigorous centrifugalisation, and, particularly, to the use of sodium oxalate or fluoride as anticoagulants, since these salts even in small amounts act as poisons and harm the red cells. In our experiments we were careful to reduce disturbance by centrifugalisation to a minimum. In most experiments, however, we used oxalates in the minimum quantity to prevent coagulation during the short interval between drawing the blood and preparing the plasma. In a great majority of the cases the plasma obtained was yellow and untinged with red, so that, as far as absence of haemolysis is an indication, the red cells were unharmed. However, to test this criticism, we drew the blood in cases 13, 34, 35 into a paraffin lined tube and centrifuged at once without any anticoagulant, in cases 37 and 40 we used heparin (a substitute for hirudin) and in cases 41, 42 and 43 potassium citrate, as recommended by Richter-Quittner. The results, as will be seen from Table II, in no wise differ from those obtained with oxalate.

It is clear from our results that normal individuals differ widely both in the total cholesterol of their plasma, and in the ratio of so-called free cholesterol to cholesterol in the form of esters.

These variations could scarcely be due to the direct absorption of cholesterol from the food into the blood plasma, since, with a few exceptions, the results were obtained from blood after a fast, or several hours after a light

meal, such as breakfast, containing but a small absolute amount of cholesterol; for even if we assume that the food contained 0·15 g. of cholesterol—which is quite an outside figure—and if we assume that the whole of this found its way into the blood stream, this would, in an individual with, say, 6 litres of blood, only increase the cholesterol by 0·00025 g. %, a figure well within the error of experiment.

Red blood corpuscles.

Richter-Quittner is probably right in his contention that the red cells contain mainly free cholesterol, and in this he is in agreement with Bloor and Knudson and other observers. The method usually adopted is to calculate the composition of the red cells from analyses of the whole blood and plasma, together with a determination of the ratio of corpuscles to plasma by the haematocrit or other method. The accumulation of experimental errors involved in such a computation is however too great to indicate, with certainty, the presence of small quantities of ester. A more satisfactory method of proving the presence of ester is a direct analysis of the *thoroughly* washed corpuscles. The cholesterol is more easily extracted from corpuscles than from plasma, and, in this respect, they resemble other tissues. The first thorough and reliable investigation of animal blood was made by Wacker and Hueck [1913] who found that in horse blood the dried red cells contained 0·36 % free and no ester cholesterol, whilst the dried white cells contained 1·729 % free and 0·04 % ester. Beumer and Bürger [1913] also found that human blood cells contain only free cholesterol, though in certain diseases traces of ester appear, but in such small amount that the authors rightly consider the values should be accepted with caution.

We made only one experiment—in the case of mixed blood No. 31—and found after elaborate washing of the corpuscles 0·0036, 0·0018 and 0·0012 % of ester cholesterol in the moist corpuscles; but as the quantities of cholesterol-digitonide weighed were only 2·0, 0·8 and 1·2 mg. respectively we cannot place much reliance on the figures, except that they indicate a small positive precipitate.

An objection might be raised against the direct method that, during the somewhat lengthy process of washing, autolytic changes may have resulted in a loss or gain of ester cholesterol, but this scarcely seems likely as calculation from whole blood and plasma points to the same results. Furthermore, a large mass of thoroughly washed corpuscles which had been kept 4 months in a stoppered bottle, and had undergone considerable autolytic change, was analysed with the following results: 13·8 g. moist material gave 45·4 mg. digitonin-compound from free cholesterol, and 2·2 mg. from ester cholesterol, the latter equivalent to 0·0039 %. Evidently the cholesterol had not been appreciably changed during autolysis. The bulk of the material (73 g.) was extracted, and the cholesterol isolated and converted into benzoate. 0·356 g. of pure benzoate, melting to a turbid liquid at 145°–146° and clearing at 178°, was obtained.

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REFERENCES.

Beumer (1923). *Z. ges. exp. Med.* **35**, 328.
Beumer and Bürger (1913). *Arch. exp. Path. Pharm.* **71**, 316.
Beumer and Lehmann (1923). *Z. ges. exp. Med.* **37**, 274.
Bloor (1916). *J. Biol. Chem.* **24**, 227.
Bloor and Knudson (1916). *J. Biol. Chem.* **27**, 107.
—— —— (1917). *J. Biol. Chem.* **29**, 7.
Channon (1925). *Biochem. J.* **19**, 424.
Chauffard, Laroche and Grigaut (1920). *Ann. Med.* **8**, 69, 149, 321.
Denis (1917). *J. Biol. Chem.* **29**, 93.
Fex (1920). *Biochem. Z.* **104**, 82.
Gardner and Fox (1921, 1). *Biochem. J.* **15**, 376.
—— —— (1921, 2). *Proc. Roy. Soc. Lond.* B, **92**, 358.
—— —— (1924). *Biochem. J.* **18**, 1058.
—— —— (1925). *Proc. Roy. Soc. Lond.* B, **98**, 76.
Gardner and Williams (1921). *Biochem. J.* **15**, 363.
Randles and Knudson (1925). *J. Biol. Chem.* **66**, 459.
Richter-Quittner (1920). *Wien. Arch. inn. Med.* **1**, 425.
Terroine (1919). Contribution à la Connaissance de la Physiologie des Substances grasses et lipoïdiques. Masson et Cie., Paris, p. 334.
Thannhauser (1923). *Z. physiol. Chem.* **127**, 278.
Wacker and Hueck (1913). *Arch. exp. Path. Pharm* **74**, 416
Windaus (1910). *Z. physiol. Chem.* **65**, 110.

XXI. STUDIES ON THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA. PART II.

THE ATTRACTION OF THE PROTEINS OF PLASMA FOR STEROLS.

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It has long been recognised that the whole of the cholesterol and cholesterol esters cannot be separated from blood plasma and serum by simple shaking with ether.

That, in native serum, the ether-soluble constituents are associated in some way with the serum proteins would appear to follow from the work of Hardy [1905], Haslam [1913] and Chick [1914], and, as they are present in relatively very small amounts they acquire the solubility properties of proteins. As Hartley [1915] has pointed out, when the conditions are reversed, traces of proteins may acquire the solubility properties of the "lipoid" with which they are associated.

As far as we are aware, Bang was the first to suggest that the cholesterol of serum may be in combination with proteins, and most probably with the globulins. A verbal communication by Bang on the subject is quoted by Thaysen [1913], and Bang [1918] gave two analyses of dog's globulin and one of human in which considerable proportions of cholesterol were found, and suggested that this might form a useful subject for further study.

In a series of papers published by Handovsky, Lohmann and Bosse [1925], Bosse [1925], Bosse and Handovsky [1925], Handovsky [1925], Handovsky and Lohmann [1925], these authors determined the total cholesterol in a large number of ox-sera, and found also that on precipitating the globulins by various salts a proportion of the whole sterol was precipitated along with the globulins. They concluded that the globulin has a greater attraction for cholesterol in serum than the albumin, and attempted to gain an idea of the quantity of cholesterol in association with the globulin, and the firmness of the union, by determining the proportion of the whole sterol which can be obtained by directly shaking out the serum with ether under rigid but arbitrary conditions.

Their figures show that when the albumin fraction of the protein is high the amount that can be directly shaken out with ether is high, but when the albumin fraction is low, relatively to the globulin, the percentage of the whole cholesterol that can be shaken out with ether is low. They concluded that in normal ox-sera about 25 % of the total cholesterol is firmly bound to globulin; the rest should be bound to phosphatides. The intensity of this union, measured by the capacity of being directly shaken out with ether, is dependent on the salt and water content of the sera. They took however no account of the fact that cholesterol is present both in the free and ester conditions. Young [1922] on the basis of his polariscopic investigation assumed that the cholesterol esters are associated in a loose way with the serum-albumin. Cholesterol is precipitated with these and can, both from the serum-albumin and also from the serum, be extracted with light petroleum or with ether. Troensegaard and Koudall [1926] obtained some evidence of the existence of cholesterol as a prosthetic group in serum-globulin, from which by simple extraction no cholesterol was obtained, by the fractionation of the acetyl bases of the different blood proteins. From serum-globulin they obtained hydrocarbons $C_{16}H_{28}$ and $C_{18}H_{32}$ which they considered must have originated from a cholesterol complex present in the globulin, since they were not obtained from either globin or albumin.

Much more work will evidently have to be done on this reaction before their conclusions can be regarded as firmly established.

In the same year Theorell [1926] analysed the proteins precipitated from horse plasma by fractional saturation with ammonium sulphate. These fractions, even after dialysis and redissolution, were found to contain considerable quantities of lipins. 77 % of the total cholesterol of the plasma was thus precipitated.

The ratios of the cholesterol to ether-soluble phosphorus of fibrinogen, globulin and albumin fractions were respectively 20·5, 16·9 and 9·7, which may be taken as an indication of the distribution of cholesterol and phosphatides between these fractions. In this connection we may point out that Wacker and Hueck [1913] in very careful experiments with horse blood showed that pure fibrin contains no cholesterol, though commercial samples do contain small amounts.

For some time past we have been working on this problem, and in view of the recent papers quoted above, we think it may be of interest to give an account of our work to date.

Cholesterol is quite insoluble in water, in alkalis and in dilute mineral acids, but Porges and Neubauer [1908] found that colloidal suspensions in water may be prepared by adding a solution of cholesterol in acetone to water and dialysing. They concluded from the properties of the fluid that it was an anodic suspensoid. This work was verified by Partington [1911] who observed that a solution of cholesterol in alcohol gave a colloidal solution when added in small quantities to water with stirring. The alcohol was removed by

dialysis and the colloidal solution was left for several months without very marked deposition. It was immediately precipitated by mineral acids, fixed alkalis and by various salts. We find that similar colloidal suspensions, though more dilute, may be made by dropping an alcohol or an acetone solution into boiling physiological saline. Similar colloidal solutions of esters, such as cholesterol palmitate, may also be readily prepared. Such colloidal solutions are markedly opalescent, whereas plasmas, containing much larger concentrations of cholesterol, are often perfectly clear and transparent to the eye, so that in plasma even if the cholesterol be not in chemical or physical union with other constituents, it must be in a quite different state to that in the artificial suspensions.

Colloidal suspensions of cholesterol, even when very dilute, we find are readily precipitated by half saturation with ammonium sulphate, and the precipitation is complete. For example, 250 cc. of a colloidal suspension of cholesterol in saline, containing 0·0055 g. cholesterol, was half saturated with ammonium sulphate, the precipitate separated and dissolved in ether and estimated by means of digitonin. It gave 0·0246 g. cholesterol digitonide, equivalent to 0·00598 g. of cholesterol. Similar colloidal suspensions of cholesterol palmitate are also flocculated by half saturation with ammonium sulphate, but we are not sure that the precipitation is quite complete. For example, 200 cc. of a suspension of palmitate on evaporation to dryness and solution in alcohol yielded 0·0211 g. cholesterol digitonide, equivalent to 0·00256 %. 250 cc. of the same suspension after half saturation with ammonium sulphate yielded a precipitate which on solution in alcohol and precipitation with digitonin gave 0·0208 g. compound, equivalent to 0·00202 %. Evidently approximately 22 % of the cholesterol palmitate was not precipitated. The actual differences in weight however are not much beyond the limits of experimental error.

If the cholesterol in plasma is in a similar state of colloidal suspension we should expect the whole or almost the whole would be precipitated along with the globulin on half saturation of the plasma with ammonium sulphate.

In order to gain some idea of the proportion of the free cholesterol and cholesterol esters thrown down on simple separation of the proteins by means of ammonium sulphate, 102 g. of plasma from a case of hyperpiesia, which analysis had shown to contain 0·087 % of free cholesterol and 0·166 % in ester form, were diluted with an equal bulk of water, and precipitated by the addition of 204 cc. of saturated ammonium sulphate. The precipitated globulins were filtered and well washed with half saturated ammonium sulphate solution. The filtrate and washings were then saturated with ammonium sulphate, and the precipitated albumins filtered and washed with a saturated solution of ammonium sulphate. The globulin precipitate was then separated into euglobulin and pseudoglobulin by digestion with saturated sodium chloride solution. The proteins thus separated were not dried and weighed, but were at once dissolved in 2 % caustic soda solution and extracted with

ether according to the method of Fex [1920]. The free and ester cholesterol were then determined in the various ether extracts, with the following results.

Table I.

Protein	Wt. of digitonin comp. in g. from		Sterols as % of original plasma			Sterols as % of cholesterol of plasma		
	Free cholesterol	Ester cholesterol	Free cholesterol	Ester cholesterol	Total cholesterol	Free cholesterol	Ester cholesterol	Total cholesterol
Euglobulin	0·0826	0·0768	0·0197	0·0183	0·0380	22·6	11·0	15·0
Pseudoglobulin	0·0648	0·1136	0·0155	0·0271	0·0426	17·8	16·3	16·8
Albumin	0·1771	0·3258	0·0422	0·0777	0·1199	48·5	46·8	47·4
			0·0774	0·1231	0·2005	88·9	74·1	79·2

The protein-free filtrates were thoroughly extracted with ether, but no sterol, either free or ester, was obtained. On boiling these filtrates with acetic acid for many hours, and re-extraction, a minute portion of cholesterol was obtained, weighing in the form of digitonin compound only 1·4 mg.

From this experiment it is clear that the whole of the cholesterol and cholesterol esters is thrown down with the proteins on precipitation with ammonium sulphate. There is a loss however of about 20 % of the sterol, in the proportion of about 1 of free to 2·3 of ester. The loss must represent sterol so intimately associated with one or other of the proteins as to be difficult of extraction by shaking the alkaline solution with ether.

Further experiments with other samples of plasma showed that by repeated re-solution and re-precipitation of the protein fractions, the protein could be largely separated from the sterol thrown down with it in the initial precipitation; and also that the difficultly extractable portions were associated rather with the globulins than the albumin.

We therefore decided to obtain a large sample of blood from a normal individual, and subject the plasma to a more thorough fractionation.

Mr C. S. Coleman, a vigorous young man aged 19, in perfect health, volunteered to give us 450 cc. of blood, and we take this opportunity of expressing to him our sincere thanks.

The plasma was light brown in colour, clear, and showed no trace of haemolysis. On analysis it was found to contain 0·0445 % free, 0·1099 % ester and 0·0057 % of cholesterol isolated only by acid hydrolysis. The total was thus 0·1602 %. 200 cc. of this plasma were diluted to 1000 cc. with water and mixed with 1000 cc. of a saturated solution of ammonium sulphate. The globulins were filtered and washed with half saturated ammonium sulphate. The filtrate and washings were then saturated with ammonium sulphate and the albumin filtered and washed with saturated ammonium sulphate. The globulins were then dissolved in physiological saline and the albumin in water, carefully filtered, and made up to 1 litre each. The globulin solution was slightly opalescent and the albumin perfectly clear and transparent. The globulin solution was then again precipitated by half saturation with ammonium sulphate and the globulin washed. The filtrate and washings were

then saturated with ammonium sulphate and the precipitated albumin, after washing, dissolved in water and added to the main albumin solution. The aqueous albumin solution was then half saturated with ammonium sulphate and a small quantity of globulin which separated was added after washing to the main globulin fraction.

The albumin solution was then saturated with ammonium sulphate and the precipitated albumin filtered and washed with saturated ammonium sulphate. This process was then repeated a third time, when the albumin fraction separated from the globulin, and the globulin separated from the albumin fraction, were practically negligible.

The globulins were then re-dissolved in saline, made up to a litre and re-precipitated by half saturation with ammonium sulphate. This process was repeated four times. The albumin was then dissolved in a litre of water, carefully filtered and re-precipitated by saturation with ammonium sulphate. This process was also repeated four times. The final albumin dissolved in water, giving a perfectly clear and transparent solution.

The final globulin precipitate was then digested over night in a considerable bulk of saturated sodium chloride, and thus approximately separated into pseudoglobulin and euglobulin. This process was not repeated more than once.

The free protein fractions were then coagulated by heat in the usual way, thoroughly washed free from salts, dried *in vacuo* over sulphuric acid and weighed.

In this way 0·7129 g. of pseudoglobulin, 2·7077 g. of euglobulin and 10·133 g. of albumin were obtained.

These proteins were allowed to soak in 2 % caustic soda solution until they swelled to jelly, and were then heated on the water-bath with a suitable further quantity of the alkali, until they went into solution, which took half to one hour. After cooling, the alkaline fluids were thoroughly extracted with ether in the manner described for plasma in Part I, and the free and ester cholesterol determined by digitonin. The extracted alkaline fluids were then evaporated to dryness and heated for 5 to 6 hours with glacial acetic acid containing hydrochloric acid. The fluid was then diluted, neutralised and extracted again with ether. After distilling off the ether, the residue was hydrolysed by means of alcoholic sodium ethoxide to saponify any cholesterol acetate. After extraction the cholesterol set free by acid hydrolysis was precipitated by digitonin.

The results are summarised in Table II.

It was noticed that the surface of the filter papers used in filtering the globulin became covered with a varnish-like substance, insoluble in physiological salt solution. The papers were therefore cut up, thoroughly washed with saline, dried and extracted in an all-glass extraction apparatus with ether. On evaporating the ethereal solution, 0·495 g. of a brownish oil was obtained. This on treatment with digitonin yielded 0·2236 g. of compound

Table II.

Protein	Wt. of protein	Cholesterol digitonide in g. from: Free cholesterol	Ester cholesterol	Cholesterol obtained by acid hydrolysis	Cholesterol (g.) per 100 g. of protein: Free	Ester	By acid hydrolysis	Total	Cholesterol (g.) per 100 g. of original plasma: Free	Ester	By acid hydrolysis	Total
Euglobulin	2·7077	0·1064	0·0709	0·0050	0·9553	0·6365	0·0449	1·6367	0·0129	0·0086	0·0006	0·0221
Pseudoglobulin	0·7129	0·0031	0·0052	0·0053	0·1057	0·1773	0·1807	0·4637	0·000377	0·000632	0·000644	0·00165
Albumin	10·133	0·0082	0·0102	0·0016	0·0197	0·0245	0·0038	0·0480	0·0010	0·0012	0·0002	0·0024
									0·0143	0·0104	0·0014	0·02615
									By analysis of plasma			
									0·0445	0·1099	0·0057	0·1601

from free cholesterol, and 0·3872 g. from ester cholesterol. The oil, therefore, contained 10·98 % of free cholesterol and 19·02 % of ester cholesterol. The albumin filter papers were also cut up and, after being thoroughly washed with water, were dried and extracted with ether. 0·41 g. of a dark brown solid grease was obtained, from which 0·0797 g. digitonin compound of the free cholesterol and 0·1417 g. from the ester cholesterol were obtained. It therefore contained 4·72 % of free cholesterol and 8·4 % of ester cholesterol.

Expressed in g. per 100 g. of the original plasma, the free cholesterol on the globulin papers was 0·0272 and the ester 0·0471, and that on the albumin paper 0·0097 and 0·0172.

Discussion of results.

Summarising the results of the above experiment:

Total sterol in 100 g. plasma		= 0·1601 g.
Found in proteins	0·0262 g.	= 0·1274 g.
Found in filter paper	0·1012 g.	
		Loss 0·0327 g.

The loss of sterol in this experiment is no doubt in the small amounts of intermediate fractions of proteins not worked up, and in the ammonium sulphate mother liquors, which, owing to their vast bulk, were not extracted with ether.

The percentage of total plasma sterol retained in proteins = 16·36

" " " euglobulin = 13·80

" " " pseudoglobulin = 1·03

" " " albumin = 1·50

Evidently the greater portion of the sterol retained is in the euglobulin, and in this our results confirm those of Handovsky.

The quantity of total cholesterol in combination with 1 g. of euglobulin is 0·0166 g.; Handovsky gives 0·0084 to 0·016 g. for ox-serum. Of the total free cholesterol retained in the proteins, 90·34 % is in the euglobulin, 2·64 % in the pseudoglobulin and 7·0 % in the albumin, whilst of the total ester cholesterol retained in the protein, 82·4 % is in the euglobulin, 6·05 % in the pseudoglobulin and 11·5 % in the albumin.

It will be noticed from these figures that the different proteins show much the same power of retention for cholesterol in the free as in the ester state. We do not know whether it would have been possible to eliminate all occluded sterol from the pseudoglobulin and the albumin by a more extended resolution and re-precipitation, but it is highly unlikely that this would have been the case with the euglobulin.

It is difficult to form a precise picture from our results, or those of other workers, as to the true relationship between the sterols and the proteins in natural plasma, but apparently there must be a close association with the euglobulin. It is not possible to conclude that the union is chemical in the ordinary sense, but probably there is (in such complex substances) really no sharp distinction between so-called physical adsorption and chemical union of associated substances.

It seems not improbable that changes in the globulin albumin ratio in plasma may have something to do with the variations in sterol content of plasma in disease, and even under normal physiological conditions. We hope however to deal with this in another communication.

REFERENCES

Bang (1918). *Biochem. Z.* **90**, 383.
Bosse (1925). *Pflüger's Arch.* **210**, 56.
Bosse and Handovsky (1925). *Pflüger's Arch.* **210**, 50.
Chick (1914). *Biochem. J.* **8**, 404.
Fex (1920). *Biochem. Z.* **104**, 82.
Handovsky (1925). *Pflüger's Arch.* **210**, 35.
Handovsky and Lohmann (1925). *Pflüger's Arch.* **210**, 59.
Handovsky, Lohmann and Bosse (1925). *Pflüger's Arch.* **210**, 63.
Hardy (1905). *J. Physiol.* **33**, 251.
Hartley (1915). *Brit. J. Exp. Path.* **6**, 180.
Haslam (1913). *Biochem. J.* **7**, 492.
Partington (1911). *J. Chem. Soc.* **99**, 318.
Porges and Neubauer (1908). *Biochem. Z.* **7**, 152.
Thaysen (1913). *Biochem. Z.* **62**, 110.
Theorell (1926). *Biochem. Z.* **175**, 297.
Troensegaard and Koudall (1926). *Z. physiol. Chem.* **153**, 111; **157**, 62.
Wacker and Hueck (1913). *Arch. exp. Path. Pharm.* **74**, 416.
Young (1922). *Proc. Roy. Soc. Lond.* B, **93**, 15.

XXII. THE EFFECTS OF CHEMICAL AND PHYSICAL CHANGES IN ENVIRONMENT ON RESTING BACTERIA.

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Prior and subsequent to the publication [Quastel, 1926] of certain views concerning the mechanism of oxidations induced by enzymes or tissues, experiments of a systematic nature have been carried out by us with the object of either substantiating or refuting these views.

Certain facts have been brought to light, one or two of which are, at present, difficult to interpret, but which in the main confirm the view that a seat of the many activations brought about by bacteria (*B. coli*) is associated with the surface structure of the cell. This does not imply, of course, that intracellular surface activations cannot occur. The facts, too, throw some light on the question of the specificity of enzyme action, and it will be shown that our views are in no way contradictory to or irreconcilable with the facts concerning specificity. Rather do they show that specificity of behaviour is to be anticipated; that it should be the rule and not the exception with biological activations.

Our experimental method, briefly, consists in exposing bacteria, whose activations of a certain number of substrates have been ascertained by the methylene blue technique, to varying physical and chemical conditions for definite periods of time. Such changes in environment produce effects on the organism which can be followed quantitatively by measuring the changes in the activations brought about by the treated organism. In this way a quantitative estimate of the effect of a certain environment on an organism can be obtained. Hitherto such an effect has been studied mainly from the point of view of antiseptic action. We find, however, that although exposure to a certain environment may have been lethal in the sense that few or none of the treated cells are capable of reproduction in the usual nutrient media, they are still capable of accomplishing a number of activations. This number diminishes and the activating power usually becomes less powerful as the length of exposure increases until eventually all activating power disappears.

Whether this experimental method will enable us to perceive precisely how an antiseptic exerts its lethal effect still remains to be seen, but it is clear that the method will allow us to compare and contrast the effects of certain lethal materials in a manner much more extensive than has hitherto been possible.

We have confined ourselves exclusively to the study of one organism, *B. coli*, four different strains having been used in the course of the work.

A stock suspension of resting *B. coli* is prepared in the following way. In each of a series of Roux bottles of 1100 cc. capacity are placed 150 cc. tryptic broth prepared according to Cole and Onslow's method [1916], and the bottles sterilised by autoclaving. Each of the contents of 10 bottles is now inoculated with 1 cc. of a fresh (18 hr.) tryptic broth culture of *B. coli* and the bottles are incubated at 37° for 40 to 48 hours. After this period the contents of the bottles are centrifuged and the clear supernatant liquid is decanted from the deposit of the organism. This deposit is suspended in 0·85 % pure salt solution and the suspension again centrifuged. This is repeated so that finally the organism has been twice washed. It is now suspended in 200 cc. of 0·85 % salt solution and vigorously aerated for 3 to 4 hours. This serves to produce a homogeneous suspension and to remove any easily oxidisable material which may be present. The bacterial suspension is placed in a flask plugged with wool and stored at 0°. Investigations with resting bacteria should be carried out within a fortnight of their preparation. After this period a new preparation should be made.

Activations by the bacteria are determined by the methylene blue method described in detail in earlier communications [Quastel and Whetham, 1925, 1, 2; Quastel and Wooldridge, 1925]. The stock suspension of bacteria, which should always be kept at 0°, may be diluted to suit the worker's needs. We find it convenient, for this work, to dilute the suspension to such an extent that 1 cc. will not reduce *in vacuo* 7 cc. 1/35,000 methylene blue solution at p_H 7·4 and 45° in one hour and will, in presence of $M/140$ sodium succinate, reduce the same quantity of methylene blue under the same conditions in approximately 10 minutes.

The actual treatment of the organism is described fully in the experimental sections which follow. After treatment the organism is well washed by centrifuging and made up to its initial concentration, previous to treatment, with normal saline solution. The activations of the treated organism are now determined in exactly the same way as for the normal organism.

I. *The variation of reduction velocity of methylene blue with quantity of organism.*

The time of reduction of a constant amount of methylene blue by a given donator is roughly inversely proportional to the quantity of organism present. This is shown by the value qt (Table I) where q = quantity of organism present and t = reduction time. The value is a fair constant with such

donators as formic acid and lactic acid. With succinic acid and glucose the value is only constant for relatively high concentrations of organism; as the quantity of organism becomes smaller the value qt rises rapidly. This divergence from the linear relation between reduction velocity and concentration of organism, as the latter approaches small values, is due to the relatively slow reduction which allows for at least two inhibiting effects on the organism to become appreciable and so to lengthen the time of reduction. These inhibiting effects are:

(1) the action of methylene blue on the organism (see Section VI);

(2) the action of temperature (45°) on the organism (see Section II).

Both the presence of methylene blue and a relatively high temperature tend, if long sustained, to produce inactivations in the organism which are relatively more marked with certain donators than with others. It is this circumstance which accounts for the relatively greater deviations from linearity exhibited by some donators than by others.

Table I.

Each vacuum tube contained 2 cc. phosphate buffer p_H 7·4, 1 cc. 1/5000 methylene blue solution, q cc. of the suspension of organism, and the donator, the volume being made up to 7 cc. with freshly boiled saline solution. Reductions were carried out *in vacuo* at 45°. *Coli* 1, *coli* 2, etc., were different suspensions and strains of *B. coli*; results with *coli* 1 can be compared strictly quantitatively with one another but only qualitatively with *coli* 2, etc. All substances under investigation were brought to p_H 7·2 by sodium hydroxide or hydrochloric acid. Controls with all organisms (absence of donators) showed no reduction within 2 hours. The reduction time t is given in minutes. In this table, as elsewhere in this paper, the sign ∞ indicates (except where otherwise stated) that reduction does not occur within 2 hours. The following are typical results.

M/140 succinic acid *Coli* 1			*M*/140 lactic acid *Coli* 1			*M*/140 formic acid *Coli* 2			*M*/140 glucose *Coli* 3		
q	t	qt	q	t	qt	q	t	qt	q	t	qt
1	25	25	0·5	27·8	13·9	0·1	33	3·3	0·5	∞	∞
1·5	9·9	14·8	0·7	19	13·3	0·2	21·4	4·3	1·0	14·7	14·7
2	6·8	13·6	1·0	13·3	13·3	0·3	15	4·5	1·5	9·75	13·6
2·5	4·9	12·2	1·2	11·5	13·8	0·4	10·9	4·4	2·0	6·5	13·0
3	4·2	12·6	1·5	9·3	13·9	0·6	7	4·2	2·5	5·5	13·7
						0·7	6·8	4·8	3·0	4·25	12·7
						1·0	4·6	4·6	—	—	—

It is very important, however, for the correct interpretation of the results which follow to bear in mind the relative values of the reduction times for, say, four donators, formic acid, lactic acid, succinic acid and glucose, with any particular organism, and the change in these values as the concentration of organism is changed (see Table II). The ratio of reduction times

$$t_{\text{formic acid}} : t_{\text{lactic acid}} : t_{\text{succinic acid}} : t_{\text{glucose}}$$

is always of the same order for a normal organism (*B. coli*) so long as the reduction time in presence of $M/140$ formic acid is not too great (not greater than 20′). Thus, experience has shown us that with any normal organism (*B. coli*) prepared in the way we have described, if $t_{\text{formic acid}} = 3'$ to $7'$ then $t_{\text{succinic acid}}$ will be about $14'$, and t_{glucose} about $7'$ and so forth. Any great deviations from this order, which are produced by a particular factor, must be due to the relatively greater action of this factor on some mechanisms than upon others; they cannot be interpreted as due to the inactivation of the organism as a whole. For instance, if for the normal organism we have

$$t_{\text{formic acid}} = 4;\ t_{\text{succinic acid}} = 14;\ t_{\text{glucose}} = 6,$$

and on treating this organism in a certain way we now find

$$t_{\text{formic acid}} = 5;\ t_{\text{succinic acid}} = 70;\ t_{\text{glucose}} = \infty,$$

this must mean that the mechanism which activates glucose is relatively more sensitive to the treatment than that which deals with succinic acid and similarly the latter is more sensitive than that which deals with formic acid. The effect cannot be interpreted as due to the inactivation of a certain proportion of the organisms *as a whole*. If, on the other hand, we find after treatment

$$t_{\text{formic acid}} = 20;\ t_{\text{lactic acid}} = 50;\ t_{\text{succinic acid}} = 120;\ t_{\text{glucose}} = 50,$$

or

$$t_{\text{formic acid}} = 40;\ t_{\text{lactic acid}} = 120;\ t_{\text{succinic acid}} = \infty;\ t_{\text{glucose}} = \infty,$$

this would indicate the improbability of a selective action and point to an inactivation of a large proportion of the organisms as a whole.

Table II.

Coli 4. Conditions as in Table I.
Reduction times with varying concentrations of organism.
∞ = not reduced in 6 hours.

q	1	1/2	1/4	1/8	1/16	1/32
Succinic acid $M/140$	17	36·5	77	390	∞	∞
Lactic acid $M/140$	8	15	34	126	250	∞
Formic acid $M/140$	3·2	4·5	10	18	42	91
Acetic acid $3M/14$	4	6	12·5	24	55	164
α-Hydroxybutyric acid $M/70$	10	20	58	∞	∞	∞
*Glycerol $M/7$	11·5	19	138	∞	∞	∞
Glutaminic acid $3M/140$	17	37	79	∞	∞	—
Glucose $M/140$	6·5	13	27	201	∞	∞
Sorbitol $M/140$	10	23·5	77	∞	∞	∞
Growth after 18 hours from one loopful of the suspension on agar	—	—	Prolific	Prolific	Prolific	Prolific

* In the case of glycerol, 1 cc. 5 % NaCl was added to the vacuum tubes.

II. *The effect of exposure of* B. coli *to varying temperatures.*

To 40 cc. of the *B. coli* suspension, 10 cc. of a phosphate (Clark and Lubs) buffer solution p_{H} 7·4 were added. The suspension was placed in a thermostat kept at the required temperature for 1 hour after it had attained this temperature. It was then surrounded by ice and the activations of the organism were determined in the usual way. For the low temperature work the organism

was placed for 1 hour in a cold room kept at the required temperature. In the case of the liquid air experiments the suspension was placed in a large test-tube and slowly immersed into liquid air. It was then withdrawn and placed immediately in another bath of liquid air for 1 hour. After transferring the tube to a beaker it was allowed to thaw at 37° and finally, on thawing, the beaker was cooled in ice. The activations of the organism were then determined. In the case of the organism exposed to 77° coagulation of the organism was observed to occur at 75°. No coagulation at lower temperatures was observed.

Results with these heated or cooled organisms are noted in Table III.

Table III.

Coli 5. Conditions as in Table I.
Reduction times after exposure to varying temperatures.

Temperature to which the organism was exposed	−180°	−21°	−6°	37°	47°	57°	67°	77°
Succinic acid *M*/140	20	19	16·7	16·2	20	24	∞	—
Lactic acid *M*/140	9	6·5	6	6	6·7	83	∞	—
Formic acid *M*/140	7	5·5	5·7	6	5·5	7	18	∞
Acetic acid *M*/4·6	12·5	8·7	8·2	7·5	8	11·7	26	∞
Butyric acid *M*/4·6	10	10·5	8	8·25	10·7	10·5	27	∞
α-Hydroxybutyric acid *M*/70	36	9·5	10	7·7	16·8	∞	∞	∞
Alanine *M*/14	—	—	—	66	∞	∞	—	—
Glutaminic acid *M*/140	—	—	—	31	63·5	∞	—	—
Glycol *M*/4·6	∞	—	—	5·5	36	∞	∞	∞
Glycerol *M*/7	∞	—	—	29·5	69·5	∞	—	—
α-Glycerophosphoric acid *M*/7	8	6	5·7	5	6·7	11·5	26·5	∞
Glucose *M*/140	∞	9	9·5	7·3	15	∞	∞	∞
Fructose *M*/140	∞	7·5	6·7	5·9	12	∞	∞	∞
Mannose *M*/140	∞	11·25	10·25	8	14·5	∞	—	—
Galactose *M*/140	∞	10	12	7·7	16	∞	—	—
Mannitol *M*/140	∞	17·5	15	11·5	27	∞	∞	∞
Sorbitol *M*/140	∞	22·5	17·7	13·5	35	∞	—	—
Fumaric acid *M*/35*	+	—	—	+	+	+	0	—
Na nitrate *M*/70*	+	—	—	+	+	+	0	—
K chlorate *M*/70*	+	—	—	+	+	+	0	—

* A + sign indicates that activation occurs, a zero sign that it has disappeared. The activation is determined by the inability of a mixture of the substance with an active donator to reduce methylene blue in presence of the organism.

Examination of the table shows that change in temperature has a selective action on the activating mechanisms of the organism. Thus we have for exposure at 37° the normal values:

$$t_{\text{formic acid}} = 6;\ t_{\text{succinic acid}} = 16{\cdot}2;\ t_{\text{lactic acid}} = 6;\ t_{\text{glucose}} = 7{\cdot}3.$$

For exposure at 57° we obtain

$$t_{\text{formic acid}} = 7;\ t_{\text{succinic acid}} = 24;\ t_{\text{lactic acid}} = 83;\ t_{\text{glucose}} = \infty.$$

As the temperature is increased we find that the most sensitive mechanisms are those for alanine, glycerol, glycol, the sugars and glutaminic acid; then come those for lactic acid, succinic acid and fumaric acid, the most resistant being those for formic, acetic, butyric, and α-glycerophosphoric acids.

Exposure of the organism for an hour to a temperature of − 21° does not appear to have an appreciable effect, but exposure to the temperature of liquid air for this time affects the organism considerably. Here again the most sensitive mechanisms are those for glycol, glycerol, the sugars and glutaminic acid. The other mechanisms are but slightly affected.

III. *The effect of exposure of* B. coli *to varying H ion concentrations.*

To 45 cc. of the suspension of *B. coli* were added 25 cc. of a buffer solution at the required p_H and 10 cc. of normal saline solution. The suspension was warmed to 37° and then placed in the incubator at 37° for 1 hour. It was centrifuged, washed with normal saline, emulsified and made up to 45 cc. with saline. The activations of the treated organism were then determined. Phthalate buffers were used for p_H 2·2, 3·0, 3·8 and 5·0; phosphate buffer for p_H 7·0 and borate for p_H 9·0 and 11·0. In one series no buffer, but caustic soda to reach a final concentration of *N*/30 was added. At p_H 2·2, 3·0 and 3·8 the organism was flocculated and difficult to emulsify after centrifuging; between p_H 5 and 11 there was no apparent change in the organism. According to Nicolai [1926] the isoelectric point of *B. coli* is at p_H 4·5. In *N*/30 NaOH the organism appeared to be partially dissolved. The results are expressed in Table IV.

Table IV.

Coli 6. Conditions as in Table I.
Reduction times after exposure to varying H ion concentrations.

p_H at which the organism was exposed ...	2·2	3·0	3·8	5·0	7	9	11	that of *N*/30 NaOH
Succinic acid *M*/140	∞	∞	83	45	14	15	43	∞
Lactic acid *M*/140	∞	∞	23·5	10·3	7	6	13	∞
Formic acid *M*/140	81	16	5·1	5	6	5·1	14·5	∞
Acetic acid *M*/14	61	17	15·5	6·6	6	6	22	∞
Butyric acid *M*/4·6	58	14	8·5	5·8	6·7	6	16·5	∞
α-Hydroxybutyric acid *M*/70	∞	∞	66	11·3	6·5	8·7	27·5	∞
Alanine *M*/14	∞	∞	∞	42	28·3	19	∞	—
Glutaminic acid *M*/140	∞	∞	∞	81	34·3	23	∞	—
Glycol *M*/4·6	∞	∞	90	51	15·3	20	90	∞
Glycerol *M*/7	∞	∞	110	76	20·2	24·7	∞	—
α-Glycerophosphoric acid *M*/7	108	13	6·2	5	5·1	4·2	14	∞
β-Glycerophosphoric acid *M*/7	∞	∞	∞	∞	31	27	∞	—
Tartaric acid *M*/4·6	∞	∞	∞	34	19	25	∞	∞
Glucose *M*/140	∞	∞	16	6·7	5·2	5·2	36	∞
Fructose *M*/140	∞	∞	16	4·5	5	5·2	28·5	∞
Mannose *M*/140	∞	∞	23·7	5·5	4·7	6·9	33	∞
Galactose *M*/140	∞	∞	17	5·7	4·8	6·3	32	∞
Xylose *M*/140	∞	∞	∞	19	5·25	8	∞	∞
Mannitol *M*/140	∞	∞	27	7·7	9·7	9·2	43	∞
Sorbitol *M*/140	∞	∞	23·5	8	8	9·2	42	∞
Fumaric acid *M*/35	0	0	+	+	+	+	+	—
Na nitrate *M*/70	0	0	+	+	+	+	+	—
K chlorate *M*/70	0	0	+	+	+	+	+	—

It will be observed that exposure to different hydrogen ion concentrations has a selective effect on the activating mechanisms of the organism.

The mechanisms most sensitive to increase in hydrogen ion concentration are those for glycerol, glycol, glutaminic acid and β-glycerophosphoric acid; then come those for the sugars and succinic acid; then that for lactic acid, and finally, the most resistant, those for formic acid, acetic acid, butyric acid and α-glycerophosphoric acid. Increase in H ion concentration has not so vigorous an effect on the sugar-activating mechanisms relative to those for succinic acid and lactic acid as has increase in temperature.

IV. *The effect of exposing* B. coli *to varying salt concentrations.*

To 70 cc. of a suspension of *B. coli* were added 40 cc. buffer solution of the appropriate p_H, the required quantity of pure sodium chloride dissolved in distilled water and sufficient water to make up the volume to 150 cc. The suspension was warmed to 37° and then incubated for 1 hour or for 24 hours at 37°. After the period of treatment the organism was washed by centrifuging, emulsified and made up to 70 cc. with normal saline solution. Its activations were determined and are noted in Table V. With the strain used for the work expressed in Table V only exposure to a concentration of 14 % NaCl produced a flocculation of the organism. It was easy to emulsify the centrifuged deposit of the treated organism.

Examination of the table shows that exposure to high salt concentrations results in a selective destruction of the activating mechanisms. Thus, whereas exposure at p_H 7·4 for 1 hour to 5 % NaCl has relatively little effect,

$$t_{\text{formic acid}} = 4{\cdot}5;\ t_{\text{lactic acid}} = 7{\cdot}5;\ t_{\text{succinic acid}} = 10;\ t_{\text{glucose}} = 8{\cdot}7,$$

exposure at p_H 7·4 for 24 hours to 5 % NaCl gives us

$$t_{\text{formic acid}} = 6{\cdot}2;\ t_{\text{lactic acid}} = 7{\cdot}7;\ t_{\text{succinic acid}} = 18;\ t_{\text{glucose}} = \infty.$$

The effect of time on the action of high concentrations of salts is very marked. The mechanisms most sensitive are, as in previous cases, those for glycerol, glutaminic acid and the sugars. Then come those for succinic acid and lactic acid, and the most resistant are those for formic acid, acetic acid, butyric acid and α-glycerophosphoric acid.

An interesting point is the distinction between fructose and glucose shown in the third and fifth columns of the table. We will notice this distinction in a number of other cases.

V. *The effect of exposure of* B. coli *to varying concentrations of nitrites.*

The treatment was carried out here exactly in the same way as that described in section IV. The results are expressed in Table VII, comparative results with similar concentrations of sodium chloride being also given. Columns K and J of Table X show results carried out with another strain of *B. coli* exposed to 5 % NaCl and to 5 % $NaNO_2$ at p_H 6·0 for 1 hour. Table VI gives results, less systematically carried out, with a different strain of *B. coli* exposed to nitrites under various conditions.

Table V.

Coli 7. Conditions as in Table I. Temperature of exposure 37°.
Reduction times after exposure to varying salt concentrations.

Conc. of NaCl to which the organism was exposed ...	*Control Normal organism*	5 %	5 %	5 %	5 %	14 %	14 %	5 %	5 %
Time of exposure in hrs. ...	—	1	24	1	24	1	24	1	24
p_H ...	7·4	5·0	5·0	7·4	7·4	7·4	7·4	9·0	9·0
Succinic acid *M*/140	9·5	23·5	40	10	18	14·5	17	22	27·5
Lactic acid *M*/140	5·7	9·5	11	7·5	7·7	7	13	11	33
Formic acid *M*/140	4·5	6	4	4·5	6·2	4	5·5	7	6·5
Acetic acid *M*/4·6	4·5	6·5	4·5	5	5	5	6·5	6·2	10·2
Butyric acid *M*/4·6	5	6	6	5·5	7	7	6·5	6·5	9·3
α-Hydroxybutyric acid *M*/70	7·5	14·5	35·7	6·5	19·5	10	24·5	13·2	105
α-Glycerophosphoric acid *M*/14	4	6	4	5	6	6	5	5	11
Glycerol *M*/7	14	∞	∞	25	∞	∞	∞	∞	∞
Glutaminic acid *M*/46	12·5	24	∞	21·5	∞	41	∞	105	∞
Glucose *M*/140	6·5	9·2	∞	8·7	∞	16·5	∞	15	∞
Fructose *M*/140	5	8	114	6·5	31	9·2	∞	11·7	∞
Mannose *M*/140	7	10	∞	9·5	∞	12·5	∞	17·5	∞
Galactose *M*/140	6·5	14	∞	8·5	∞	13·2	∞	18	∞
Mannitol *M*/140	9·2	17·5	∞	12	∞	20·5	∞	27	∞
Sorbitol *M*/140	10	17·5	∞	10·5	∞	19	∞	25	∞
Fumaric acid *M*/35	+	+	+	+	+	+	+	+	+
Na nitrate *M*/70	+	+	+	+	+	+	+	+	+
K chlorate *M*/70	+	+	+	+	+	+	+	+	+

Table VI.

Conditions as in Table I. Temperature of exposure 37°.

Coli	Concentration of $NaNO_2$ to which the organism was exposed %	p_H of exposure	Time of exposure hrs.	$t_{glucose}$ mins.	$t_{succinic\ acid}$ mins.	$t_{lactic\ acid}$ mins.	$t_{formic\ acid}$ mins.
9	1	6·8	22	60	∞	12·5	3·8
9	1	6·8	46	∞	∞	18	26
10	5	7·0	20	∞	∞	60	8
10	2	6·3	24	—	∞	33	54
11	1	6·8	30	—	∞	19	58

It will be observed that the tendency of the effect of nitrites is towards selectivity of action, though this is not always well marked. Nitrites at a low p_H are more destructive than those at a higher p_H, as would be anticipated if nitrous acid were the destructive agent. The very clear difference between the effects of nitrites and those of NaCl under the same conditions shows that the action of the former is not entirely an osmotic one.

As to selectivity of action, that due to nitrites seems to be similar in its general tendency to the action brought about by exposure to increased hydrogen ion concentrations (see Table IV). If indeed an amino-group plays an essential part in the activations, this similarity of action is to be anticipated.

Table VII.

Coli 8. Conditions as in Table I, except that in columns *A* and *B* each vacuum tube contained 1 cc. 5 % NaCl. In every case in this table, as in Tables VIII, IX and X, the glycerol tube contained 1 cc. 5 % NaCl. This was found to be beneficial, with glycerol, in accelerating the last stage of the reduction. Temperature of exposure 37°.

Reduction times after exposure to varying NaCl and $NaNO_2$ concentrations.

Character of exposure ...	*A* Control Normal organism	*B* 5 % NaCl	*C* 2 % NaCl	*D* 2 % $NaNO_2$	*E* 1 % NaCl	*F* 1 % $NaNO_2$	*G* 1 % NaCl	*H* 1 % $NaNO_2$	*J* 1 % NaCl and 1 % H.COONa	*K* 1 % NaNO and 1 % H.COONa
Time of exposure in hrs. ...	—	1	1	1	1	1	23	23	23	23
p_H of exposure	—	5·0	5·0	5·0	6·0	6·0	6·0	6·0	6·0	6·0
Succinic acid *M*/140	17	121	60	∞	16·2	∞	22	∞	55	∞
Lactic acid *M*/140	8·7	47	18	107	8·7	24	11	∞	16·5	77
Formic acid *M*/140	4	5	4	27	3	4·2	3·2	52	5	∞
Acetic acid *M*/46	4·7	5·7	3·2	27·5	3·7	5·7	4	∞	5·7	∞
Butyric acid *M*/46	6	7	6	41	4·2	6·7	5	∞	6	∞
α-Hydroxybutyric acid *M*/70	10	35	17	∞	10·5	34·2	20·7	∞	36·2	∞
α-Glycerophosphoric acid *M*/14	11	13·5	6	∞	5·5	7	4·2	—	7·2	∞
Glycerol *M*/7	13·5	80	29·5	∞	27	46	46·2	∞	76	∞
Glutaminic acid *M*/46	21·2	∞	85	∞	16·5	125	61	∞	∞	—
Glucose *M*/140	7	15	9·5	∞	7·2	14·2	13	∞	24	∞
Fructose *M*/140	7·5	18	8·5	∞	5·2	15·5	10	∞	21	∞
Mannose *M*/140	8	17	8	—	6	15·7	13·2	—	30·5	—
Galactose *M*/140	7	15·7	10	—	6·5	15·5	12	—	26	—
Mannitol *M*/140	11·2	41	17	∞	8	20	18·5	∞	34	—
Sorbitol *M*/140	10·5	28·5	17·5	—	10	25·7	20	—	37	—
Fumaric acid *M*/35	—	—	—	—	+	0	—	—	—	—
Na nitrate *M*/70	—	—	—	—	+	+	—	—	—	
K chlorate *M*/70	—	—	—	—	+	+		—	—	—

We have not observed any protective action by formates on the formic acid-activating mechanism in *B. coli* when this is exposed to a mixture of nitrites and formates. A protective action has been found by Myrbäck [1926] in the case of invertase, by the presence of sucrose, when this enzyme is exposed to nitrous acid. Inspection of columns *B* and *C* of Table IX and of column *K* of Table VII shows that no such protective action by formates occurs, under our experimental conditions, with *B. coli*.

On exposure of the organism, *coli* 8, to 2 % NaCl at p_H 5·0, and to 1 % NaCl at p_H 6·0 flocculation of the organism occurred. Under the same conditions no appreciable flocculation occurred when $NaNO_2$ was substituted for NaCl; a slight flocculation was observable only in the case of exposure for 23 hours to 1 % $NaNO_2$ at p_H 6·0. Since the effects of the same concentration of salt on different strains vary somewhat, quantitatively, it is necessary when observing the effects of nitrites to perform comparative experiments with equivalent amounts of salt.

VI. *The effect of methylene blue on* B. coli.

To 12 cc. of the suspension of *B. coli* were added 12 cc. phosphate buffer p_H 7·4 and 12 cc. 1/5000 methylene blue solution. The suspension was allowed to stand in a thermostat at 45° for $1\frac{3}{4}$ hours. After this period it was cooled in ice and 3 cc. of the mixture were added to a vacuum tube containing 1 cc. phosphate buffer p_H 7·4, 1 cc. of the donator solution and 2 cc. normal saline. The reduction times for the methylene blue were then determined at 45° in the usual way. The following results were obtained:

$$t_{\text{succinic acid}} = \infty;\ t_{\text{lactic acid}} = 43;\ t_{\text{formic acid}} = 7;$$
$$t_{\text{glucose}} = 50;\ t_{\text{sorbitol}} = 52.$$

For *B. coli* treated similarly but in the absence of methylene blue we obtained:

$$t_{\text{succinic acid}} = 18;\ t_{\text{lactic acid}} = 9;\ t_{\text{formic acid}} = 4;$$

$$t_{\text{glucose}} = 6;\ t_{\text{sorbitol}} = 9{\cdot}2.$$

Table VIII.

Coli 14. Conditions as in Table I. p_H of exposure = 7·4.

	Reduction times.										
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*J*	*K*	*L*
Character of exposure ...	*Control Normal organism*	Toluene	10 % *cyclo*-Hexanol	10 % *cyclo*-Hexanol	Ether	"Colloidal iron"	"Colloidal iron" and toluene	Methylene blue at 45°	Control on *H* at 45°	15 % $CHCl_3$	15 % $CHCl_3$
Time of exposure ...	—	5 mins.	5 mins. Not removed	5 mins. Removed	10 mins.	5 mins.	5 mins.	2 hrs.	2 hrs.	30 mins.	18 hrs.
Succinic acid *M*/140	9·2	10·2	—	∞	16	14	16·7	163	20	59	80
Lactic acid *M*/140	5·7	6·7	∞	∞	11·2	10	16	49	12	11	14·5
Formic acid *M*/140	3	3	∞	15·2	3·7	4·5	5·5	5·5	4·2	6·2	11
Acetic acid *M*/4·6	3·7	4·5	∞	49·5	6	5·2	8·5	8	6·2	8·7	8·7
Butyric acid *M*/4·6	5·7	5·2	∞	28	5·7	6·2	5	6·5	5·7	—	—
α-Hydroxybutyric acid *M*/70	7·7	23·5	—	∞	40	13	44	∞	25	51	70
α-Glycerophosphoric acid	3·7	4·5	∞	14·5	5	5·2	8	—	—	7	7
β-Glycerophosphoric acid	6	∞	∞	66	—	—	—	—	—	—	—
Glycerol *M*/7	10	∞	—	∞	∞	17	∞	∞	29	∞	∞
Glutaminic acid *M*/46	11·7	∞	—	∞	∞	19	∞	∞	31·5	∞	∞
Glucose *M*/140	4·5	∞	—	∞	∞	8	∞	∞	10	∞	∞
Fructose *M*/140	4	107	—	∞	129	8·5	∞	∞*	10·5	115	∞
Mannose *M*/140	4·2	∞	—	∞	∞	9·5	∞	∞	11·5	∞	∞
Galactose *M*/140	4·5	∞	—	∞	∞	8	∞	∞	13·2	∞	∞
Mannitol *M*/140	9·5	∞	—	∞	∞	16	∞	∞	16	∞	∞
Sorbitol *M*/140	7	∞	—	∞	∞	17	∞	∞	21	∞	∞
Fumaric acid *M*/35	—	+	—	0	+	+	—	+	—	+	+
Na nitrate *M*/70	—	+	—	+	+	+	—	+	—	+	+
K chlorate *M*/70	—	+	—	+	+	+	—	+	—	+	+
Growth on agar from a loopful of treated organism	Prolific	Few discrete colonies	—	Few discrete colonies	Few discrete colonies	Prolific	Few discrete colonies	Few discrete colonies	Prolific	None	—

* This tube reduced completely in 5 hours whereas the other sugars showed no reduction in this time.

Table VIII, column *H*, gives results with another strain of *B. coli* treated for 2 hours with methylene blue in a similar manner to the above. The results with the organism treated similarly but in the absence of the dyestuff are given in column *J*.

It will be observed that a selectivity of action is demonstrable. As before, the most resistant mechanisms are those for formic acid, acetic acid, and butyric acid. The sugars, glycerol, glutaminic acid and succinic acid systems are among the least resistant. The organism treated with methylene blue on subculture gave rise to a few discrete colonies, whereas the control gave a prolific growth.

VII. *The effects of toluene, benzene and phenol on* B. coli.

To 25 cc. of the suspension of *B. coli* were added 5 cc. phosphate buffer solution p_H 7·4 and 5 cc. toluene. The mixture was shaken, allowed to stand for 5 minutes at room temperature and then centrifuged. The deposit of organism was washed and made up to 25 cc. with normal saline solution. The organism was treated in the same way with benzene. Results with

Table IX.

Coli 12. Conditions as in Table I. Concentrations of substrates as in Table VIII.

Reduction times.

Character of exposure ...	*A* *Control Normal organism*	*B* 1 % NaCl + 1 % $NaNO_2$	*C* 1 % H.COONa + 1 % $NaNO_2$	*D* 1 % NaCl	*E* 1 % H.COONa	*F* Toluene	*G* C_2H_5OH	*H* $CHCl_3$	*J* *cyclo*-Hexanol	*K* *cyclo*-Hexanol	*L* $CHCl_3$
Time of exposure ...	—	17 hrs. p_H 7·0	17 hrs. p_H 7·0	17 hrs. p_H 7·0	17 hrs. p_H 7·0	5 mins	30 mins.	4 hrs.	Not removed	Removed after 5 mins.	24 hrs.
Succinic acid	14·2	∞	∞	34	32·5	21·5	∞	69	∞	∞	∞
Lactic acid	7·7	54	41·5	17	13·5	8·7	25	10	∞	∞	13
Formic acid	4	14·2	∞	7·2	6·2	4·7	25·7	9·2	∞	49	21·5
Acetic acid	5·5	15·2	∞	7·5	7	6·5	19·7	20·2	∞	106	22·5
Butyric acid	7·5	20·7	∞	9·2	8·5	7	21	15·5	∞	—	61
α-Hydroxybutyric acid	10·5	∞	114	25	28	45	∞	38	∞	—	∞
α-Glycerophosphoric acid	5·5	37	∞	9	8·5	7·2	20	9·5	∞	—	14
β-Glycerophosphoric acid	9·2	—	—	—	—	∞	—	—	—	—	—
Glycerol	11·2	∞	∞	19·2	29	∞	∞	∞	—	—	—
Glutaminic acid	14·5	72	—	—	—	∞	∞	∞	∞	—	—
Glucose	5·2	55	114	9	13	∞	∞	∞	∞	∞	—
Fructose	5·5	—	69	10·5	8	∞	∞	∞	∞	∞	—
Mannose	—	—	—	—	—	∞	∞	∞	—	—	—
Galactose	—	—	—	—	—	∞	∞	∞	—	—	—
Mannitol	7·2	∞	115	—	12·5	∞	∞	∞	∞	—	—
Sorbitol	—	—	—	—	—	∞	∞	∞	—	—	—
Fumaric acid	—	?	0	—	—	+	0	+	—	—	—
Na nitrate	—	+	+	—	—	+	+	+	—	—	—
K chlorate	—	—	—	—	—	+	+	+	—	—	—

Table X.

Coli 15. Conditions as in Table I. p_H of exposure 7·4. Concentrations of substrates as in Table VIII.

Reduction times.

Character of exposure ...	*A* *Coli* 12	*B* Thorough extraction with ether	*C* 1 % C_2H_5OH	*D* 7 % C_2H_5OH	*E* 14 % C_2H_5OH	*F* 15 % benzene	*G* 21 % acetone	*H* 1 % phenol	*J* 5 % NaCl p_H 6·0	*K* 5 % $NaNO_2$ p_H 6·0
Time of exposure	30 mins.	—	5 mins.	5 mins.	30 mins.	1 min.	60 mins.	5 mins.	60 mins.	60 mins.
Succinic acid	115	23·5	11·7	27·7	42	12·7	20	44	16·2	∞
Lactic acid	11·2	12	6·5	9·2	8·5	9	9	7·5	11	34
Formic acid	4·5	3	2·5	3·2	3·2	2·5	2·7	4·2	3·5	7·5
Acetic acid	5·7	3·2	—	4·2	4	3	3·7	6	4·2	17
Butyric acid	7·7	3·7	—	5	5	5	4·2	3·7	4	16·5
α-Hydroxybutyric acid	58	27	—	21·5	46	20·7	19·5	34·5	12·7	80
α-Glycerophosphoric acid	7·7	3·7	—	4·7	4·7	3·5	4·5	4·7	5·2	43
β-Glycerophosphoric acid	110	∞	—	117	∞	—	∞	∞	—	∞
Glycol	—	—	—	—	—	—	∞	∞	65	∞
Glycerol	∞	∞	—	58	∞	∞	∞	∞	15	∞
Glutaminic acid	∞	∞	—	14·5	∞	∞	∞	∞	20·5	∞
Glucose	∞	∞	5·2	15	∞	∞	41·5	∞	7	∞
Fructose	161	101	—	11	∞	67	15	147	7·2	124
Mannose	∞	∞	—	11·5	∞	∞	58	∞	6·5	∞
Galactose	∞	∞	—	11·5	∞	∞	60	∞	8	∞
Mannitol	∞	∞	—	19·5	∞	∞	∞	∞	15·5	∞
Sorbitol	∞	∞	—	20·5	∞	∞	∞	∞	16	∞
Fumaric acid	+	+	—	—	—	+	—	+	—	0
Na nitrate	+	—	—	—	—	+	—	+	—	+
K chlorate	+	—	—	—	—	+	—	+	—	+
Growth on agar from a loopful of the treated organism	—	—	Prolific	Prolific	Few discrete colonies	None	Good	Few discrete colonies	—	—

toluene are given in column *B*, Table VIII, and column *F*, Table IX those with benzene are given in column *F*, Table X.

The most striking effect of these substances after 5 minutes' treatment is the elimination of the mechanisms dealing with the sugars (though that dealing with fructose shows relatively considerable resistance), with glycerol and with glutaminic acid. The mechanisms for succinic, lactic, formic, acetic, butyric and α-glycerophosphoric acids appear to be left almost intact. Subculture of the treated organism shows that the treatment has been practically completely lethal—only a few discrete colonies are obtained.

In the case of phenol, 25 cc. of the suspension of *B. coli* were treated with 1 % neutralised phenol in presence of phosphate buffer p_H 7·4 for 5 minutes at room temperature, centrifuged and washed with saline. The washed organism was made up to 25 cc. with normal saline. Its activations are noted in column *H* of Table X. It will be observed that the same order of sensitivity obtains as with benzene and toluene; the succinic acid mechanism appears to be somewhat affected. Subculture shows that the treatment has been almost completely lethal.

VIII. *The effects of ether and chloroform on* B. coli.

B. coli was treated with ether and chloroform in the same way as described for benzene and toluene. The time of treatment with ether was 10 minutes (column *E*, Table VIII) and with chloroform 30 minutes (column *K*, Table VIII) and 18 hours (column *L*, Table VIII). With another strain (*coli* 12) the time with ether was 30 minutes (column *A*, Table X) and with chloroform 4 hours (column *H*, Table IX) and 24 hours (column *L*, Table IX). The same effects as as with toluene and benzene are obtained. It is apparent that with lapse of time the more resistant mechanisms disappear.

The following experiment was carried out with ether. 100 cc. of a thick freshly prepared suspension of *B. coli* were extracted three times with ether. The ether extracts were collected, and evaporated to dryness *in vacuo* at 20°. The activations of the extracted organism, which was washed twice with normal saline, are noted in column *B*, Table X. Half of the quantity of the extracted organism was added to the vessel containing the slight residue from the ether extract and the suspension was incubated at 37° for 2 hours. The activations of the incubated organism for succinic acid and glucose were then determined. It was found that the mechanism for glucose was still absent and that for succinic acid had disappeared. The other half of the quantity of extracted organism was allowed to stand in the incubator at 37° for 16 hours after which time it was found that the succinic acid mechanism had been eliminated.

IX. *The effect of acetone on* B. coli.

If a suspension of *B. coli* be shaken with small concentrations of acetone, no appreciable effects occur. If the acetone concentration be increased, selective

effects become apparent. Column *G*, Table X, gives results obtained with *B. coli* treated with 21 % acetone.

The results with acetone are interesting in showing a selective behaviour with the sugars. Thus the mechanism for fructose is most resistant, as is usually the case, whilst those for glucose, mannose and galactose are more resistant than those for the polyhydric alcohols. On the whole the effects of high concentrations of acetone are similar to those of chloroform, ether, etc.

X. *The effects of propyl alcohol and* cyclo-*hexanol on* B. coli.

It has been shown by Quastel and Whetham [1925, 1, 2] that the presence of propyl alcohol in the vacuum tube at a concentration of 20 % will prevent any reduction of methylene blue by donators in presence of *B. coli*. Similarly, *cyclo*-hexanol has a powerful inhibiting action on reductions in the presence of *B. coli*. It was interesting to see whether this action of propyl alcohol and *cyclo*-hexanol was reversible, *i.e.* whether after exposure of *B. coli* to such concentrations of alcohol, which in the vacuum tubes are entirely inhibitive to all reductions, activations can still occur.

To 50 cc. of *B. coli* suspension were added 20 cc. propyl alcohol and the suspension shaken and allowed to stand at room temperature for 30 minutes. (This concentration of propyl alcohol in the vacuum tube is sufficient entirely to inhibit reductions due to any of the donators we use.) Flocculation of the organism occurred. It was washed by centrifuging and suspended in 50 cc. normal saline. The activations of the treated organism are shown in column *G*, Table IX. It will be observed that there is reversibility with the mechanisms dealing with lactic, formic, acetic, butyric and α-glycerophosphoric acids.

The effects of exposing *B. coli* to 1 % propyl alcohol for 5 minutes with subsequent washing are shown in column *C*, to 7 % propyl alcohol for 5 minutes in column D, and to 14 % propyl alcohol for 30 minutes in column *E* of Table X. Exposure to the last concentration of alcohol resulted in elimination of the activation of glycerol, the sugars and glutaminic acid; at this concentration, too, there was an almost completely lethal action, for subculture showed but few colonies. Exposure to the previous two concentrations which did not affect the sugar activations appreciably had but little lethal action, for subculture yielded prolific growths. Although exposure to 7 % propyl alcohol with subsequent removal of the alcohol did not result in elimination of the glucose-activating mechanism, the same concentration in the vacuum tube entirely inhibited reduction due to glucose. Thus a reversibility in the case of glucose is apparent.

The partially reversible action with propyl alcohol is also exhibited by *cyclo*-hexanol. Column *C*, Table VIII, and column *J*, Table IX, give results with 10 % *cyclo*-hexanol present in the vacuum tube; column *D*, Table VIII, and column *K*, Table IX, show the results obtained after exposure of *B. coli* to the *cyclo*-hexanol for 5 minutes with subsequent washing. The effect of *cyclo*-hexanol is more vigorous and less easily reversible than that of propyl alcohol.

XI. *The effect of "dialysed iron" on* B. coli.

10 cc. of commercial "dialysed iron[1]" was diluted to 100 cc. with distilled water, 2 drops of dilute ammonia added and the solution filtered, the filtrate now having a neutral reaction. 10 cc. of this solution were added to 50 cc. of a suspension of *B. coli* and the mixture was allowed to stand 1 hour, at the end of which time the organism had entirely flocculated, adsorbing the "iron." It was centrifuged and the deposit washed. The washed deposit was suspended in 50 cc. saline. The activations of the treated organism are noted in column *F*, Table VIII, and it will be observed that there has been but little interference by the adsorbed "iron" with the activations of the organism. That the adsorbed "iron" does not protect the organism appreciably is shown by the following experiment. 20 cc. of the "iron"-treated organism were treated with toluene in the manner described earlier. After washing, its activations were determined and are noted in column *G*, Table VIII. The effects of toluene on the treated organism are the same as those in the absence of adsorbed "iron" on the organism.

XII. *The action of sodium hydrosulphite.*

We have now made a number of attempts to restore the activating mechanisms of *B. coli* which have been eliminated by a particular treatment. Though partial reversibility occurs in the cases of propyl alcohol and *cyclo*hexanol we have had but little success with the more deep-seated changes which occur with other agents.

An effect, however, with sodium hydrosulphite on the nitrite-treated organism is curious and although it has not yet been fully investigated it is, we think, worthy of description here.

To 175 cc. of a suspension of *B. coli* were added 150 cc. phosphate buffer p_H 5·0 and 25 g. of sodium nitrite dissolved in 175 cc. distilled water. The suspension was allowed to stand in the incubator for 1 hour after which it was centrifuged, the deposit of organism washed twice and suspended in 175 cc. saline.

The activations of the treated organism were determined, the results being noted in column *A* of Table XI. It will be seen that only feeble activations of lactic, formic, acetic and α-hydroxybutyric acids remain and that for α-glycerophosphoric acid has disappeared (*i.e.* its reduction time exceeds 3 hours). To 35 cc. of the nitrite-treated organism were then added a solution consisting of 25 cc. borate buffer solution p_H 9·0 containing 2 g. sodium hydrosulphite and 2 cc. *N* NaOH solution. This suspension was allowed to stand in the incubator for 19 hours, after which it was centrifuged, twice washed and made up with normal saline to 35 cc. On determining the activations of the organism so treated it was found (see column *B*, Table XI, where all the results

[1] According to Böhm [1925] the particles in the well-known sol prepared by hydrolysing $FeCl_3$ are mostly a basic iron chloride.

are noted) that the organism had regained some of its power to activate formic, acetic and α-glycerophosphoric acids. The activity for lactic acid was somewhat diminished whilst that for the other donators had not re-appeared. When this partially restored organism was once more treated with nitrites the activities for all donators disappeared (column *C*, Table XI), and on re-treating with sodium hydrosulphite in the same way as before there was a slight restoration of the formic, acetic and α-glycerophosphoric acid mechanisms. A control experiment (absence of hydrosulphite) showed no restoration whatever of the mechanisms eliminated by nitrites.

Table XI.

Conditions as in Table I. Concentration of substrates as in Table VIII. Reduction times. ∞ =not reduced in 3 hours.

	A	*B*	*C*	*D*
Succinic acid	∞	∞	∞	—
Lactic ,,	50	72	∞	Not reduced in 6 hrs.
Formic ,,	65	9	∞	127
Acetic ,,	105	15	∞	153
Butyric ,,	59	∞	—	—
α-Hydroxybutyric acid	85	∞	—	—
α-Glycerophosphoric acid	∞	10	∞	185
Glycerol	∞	∞	—	—
Glycol	∞	∞	—	—
Glutaminic acid	∞	∞	—	—
Glucose	∞	∞	—	—
Fructose	∞	∞	—	—
Mannose	∞	∞	—	—
Galactose	∞	∞	—	—
Mannitol	∞	∞	—	—
Sorbitol	∞	∞	—	—
Fumaric acid	0	0	—	—
Na nitrate	+	+	—	—
K chlorate	+	+	—	—

We have observed with another strain of *B. coli* the same restoring action of sodium hydrosulphite. 2 % cysteine, if used for a similar period at p_H 7·4, appears to have a similar restoring action.

We do not think, however, that we have yet obtained the best conditions for maximum restoration. We have never observed any sign of restoration with an organism whose activating mechanisms have been entirely destroyed by heat.

XIII. *Experiments of a preliminary nature on* B. coli.

A number of experiments of a preliminary (*i.e.* not of a systematic) nature have been carried out in connection with the reactions of *B. coli* and their results are summarised below.

1. If a suspension of *B. coli* be treated with copper sulphate solution for half an hour at a concentration of 1/5000 and the organism is well washed, the activations for glucose, succinic acid, lactic acid and formic acid are eliminated.

If now the treated organism be exposed to a current of H_2S for 4 minutes

and the organism (which appears black) be washed free from H_2S its subsequent reactions are as follows:

$$t_{\text{glucose}} = 45;\ t_{\text{succinic acid}} = \infty;\ t_{\text{lactic acid}} = 30;\ t_{\text{formic acid}} = 12.$$

The treatment with H_2S has resulted in the restoration of a number of the activating mechanisms.

A similar effect occurs on exposing B. *coli* to mercuric chloride solution (1/5000) for the same period. After treatment we obtain

$$t_{\text{glucose}} = \infty;\ t_{\text{succinic acid}} = \infty;\ t_{\text{lactic acid}} = 65;\ t_{\text{formic acid}} = 5{\cdot}2.$$

On treating this organism with H_2S and after subsequent washing, the activations are

$$t_{\text{glucose}} = 28;\ t_{\text{succinic acid}} = \infty;\ t_{\text{lactic acid}} = 22;\ t_{\text{formic acid}} = 6{\cdot}5.$$

The action of H_2S in restoring activity of a number of mechanisms inactivated by copper or mercury is similar to the restoring action of H_2S on invertase inactivated by silver [Myrbäck, 1926].

2. If to a suspension of *B. coli* glucose be added and the solution incubated at 45° for 2 hours, the reaction of the solution becomes distinctly acid, presumably due to the formation of lactic acid by glycolysis. Taking the fall of p_H as an indication of glycolysis we find that, whenever the organism is so treated that the activating power for glucose disappears, the glycolytic power of the organism also disappears. We have treated the organism in many different ways and have always observed this parallel disappearance of glycolytic power and glucose-activating power. The inference would appear to be that the two mechanisms—glycolysis and glucose activation—are controlled in *B. coli* by the same agency, or that a direct consequence of glucose activation by *B. coli* is glycolysis.

3. We have observed on several occasions that *B. coli* which has been so treated that its activity for glucose has disappeared is still capable of activating hexosediphosphate as a donator. There is, thus, a distinct difference between these two substances in relation to the activating power of *B. coli*.

We wish to emphasise, however, that the observations in this section are of a preliminary nature and have not yet been systematically investigated.

Discussion.

In a previous paper [Quastel, 1926] the view was put forward that the activations of substrate molecules (hydrogen donators or acceptors) were due to polarisations of these molecules effected at locally intense electric fields whose presence and nature were dependent upon the characters of a surface structure. Such a polarisation implied the attachment (not necessarily permanent) of a substrate molecule to a particular grouping orientated in the surface, the field due to this grouping bringing about, provided that the strength of the field were sufficiently high, an activation of the substrate molecule.

Evidence was given to show that the chief site of reduction of methylene

blue by substrates activated by *B. coli* was at the cell surface and further evidence was brought to show that, although activations of substrates may occur within the cell, such activations could not account for the entire reduction of methylene blue, and hence a site of activation of the substrate molecules was also at the cell surface. Upon the character of this surface as well as upon the chemical and electrical nature of the substrate molecules depended the variety of activations to which *B. coli* gives rise.

When activation of a substrate molecule (H donator) occurs, the following equations represent the events which follow:

$$D \rightleftarrows A_a'' + 2H^{\cdot}$$
$$A_a \rightleftarrows A,$$

where D is the hydrogen donator, A is the oxidised form after loss of 2H and A_a is the activated form of the molecule which can only exist as such in presence of the polarising field. The act of oxidation consists in the transference of electrons from A_a'', the electrically neutral molecule A_a being transformed into the normal form A out of contact with the field. The system thus becomes amenable to the same theoretical treatment as any system which gives rise to a reduction or oxidation potential. If A is a molecule whose oxidised form is AO, then, in presence of the ions of water, it may act as a H donator in the following way:

$$A \rightleftarrows A_a$$
$$A_a + 2H^{\cdot} + 2OH' \rightleftarrows A_a(OH)_2'' + 2H^{\cdot}$$
$$A_a(OH)_2 \rightleftarrows A(OH)_2 \text{ or } AO.$$

It follows from this that given a substrate activated by the cell to form a hydrogen donator, any hydrogen acceptor will react with it so long as the acceptor is already active (gives a reduction or oxidation potential), the equilibrium point being determined by the potentials of the two systems. Thus the cell (or enzyme) will evince no specificity to such acceptors. Similarly if a substrate is activated by the cell to form a hydrogen acceptor, this will react with H donators so long as the latter are already active (give reduction potentials) and no specificity will be evinced to such donators. Certain other considerations which will be described, are, however, to be taken into account when considering the question of specificity.

The conception of the mechanism of activation stated above does not imply that an electric field, at the surface, will activate *any* molecule which comes into contact with it. If such were the case, there could be none of the selectivity of destructive action, due to exposure to abnormal conditions, which actually occurs. What we actually expect, however, is that certain fields will be able to activate a series of molecules *A*, *B*, *C* ... *X*, whilst other fields, of weaker intensity, will only be able to activate the series, say, *C* ... *X*. This follows from the fact that the activation of a certain molecule necessitates the uptake of a critical amount of energy. Thus, if a surface structure possess

two fields P and Q, the first of which activates molecules *A*, *B* and *C* and the second (the less intense) only *C*, then if this structure be attacked in some way, the strength of Q may so diminish that it can no longer activate *C* whilst that of P, diminishing proportionately, may still retain sufficient power to activate *B* and *C* but not *A*. In this way selectivity of action by a surface structure may occur.

Apart, however, from this consideration a number of other factors playing a part in the phenomena of specificity must be taken into account. There is, for instance, the sign of the polarising field and, equally important, a set of factors which are perhaps best embraced by the term "accessibility." The most obvious of these factors is the stereochemical. If, for instance, a definite orientation of the substrate molecule at the surface structure is required for activation to occur, then the stereo-structure (and polarity) of the substrate molecule will be an all-important factor in determining such an orientation and hence an activation. Thus, if there are two groupings P and Q at a surface, both of which are capable, through the intensity and nature of their fields, of activating a molecule *A*, the character of the surface at P may be so different from that at Q, that the required orientation of *A* may occur at P but not at Q. In this way a sort of "lock and key" specificity is obtained. Again, one grouping P may be capable of activating two molecules *A* and *B*; yet the polarity and stereo-structure of the two molecules may be such as to favour the accessibility of *A* to this grouping and hence its activation but not that of *B*. Hardy and Doubleday's work [1921, 1922] on lubrication serves to indicate the importance of polarity in a molecule in determining its orientation at a surface or its accessibility to a structure.

There is another factor of some importance. If in presence of a H donator, activated by an enzyme, there is present either of two acceptors both of which give the same reduction potentials, the final equilibrium point (assuming reversibility) will be the same in both cases. The rate at which this point is achieved by the two systems may, however, be greatly different.

This is indicated in Table XII where it is shown that the velocity of reduction of different dyestuffs is greatly different from what would be anticipated if the velocity of reaction were proportional to the difference in reduction potential between the reacting substances. This has also been shown by Dixon [1926] in the case of xanthine oxidase. It is easy to see why this is so, for the slow reducing indicators are the acid dyestuffs which are not so accessible to, or not so easily adsorbed by, the negatively charged cell as the more basic compounds. If the reduction occurs chiefly at the cell surface, then since the velocity of reduction is governed by the concentration of indicator as well as by the concentration of activated donator at the surface, it follows that "accessibility" of the indicator is a determining factor in controlling the velocity. Similarly, the same type of "accessibility" of a substrate molecule which has to be activated may be a determining factor in controlling the velocity of reaction. In certain cases the velocity of the latter may be so small

as to give the appearance of inactivity and we should conclude that an enzyme capable of activating the substrate in question was absent. This would be a legitimate conclusion so long as it were understood that the sole criterion for specificity of action is the velocity of reaction.

Table XII.

Coli 13. Conditions as in Table I, but with different dyestuffs as H acceptors. ∞ =not reduced within 4 hours.

Dyestuff *M*/1880	$t_{formic\ acid}$	$t_{lactic\ acid}$	$t_{succinic\ acid}$	$t_{glucose}$	Approx r_H range
Indigo carmine	∞	∞	∞	∞	7·2–11·2
K indigotintrisulphonate	∞	∞	∞	∞	8·3–12·3
K indigotintetrasulphonate	∞	∞	∞	∞	10 –14
Methylene blue	4·7	7·5	9·5	5·5	13 –17
1-Naphthol-2-sulphonate-2 : 6-dichlorophenol	180	180	∞	150	15·2–19·2
1-Naphthol-2-sulphonate-indophenol	130	118	183	125	15·4–19·4
o-Cresol-2 : 6-dichloroindophenol	45·5	42·5	55·5	43	17·3–21·3
o-Cresol-indophenol	19·5	13·1	13·2	11·5	17·7–21·7
2 : 6-Dibromophenol-indophenol	36·2	32	45	41	18·3–22·3
o-Chlorophenol-indophenol	16·5	13	13·7	14	19 –23

The results given in this paper show very clearly how certain activating mechanisms can be regarded as more resistant, others less resistant to exposure to abnormal conditions. As the time of exposure increases, there is a gradual, "step by step" disappearance of all the mechanisms. The order of sensitivity or disappearance is, on the whole, the same. Even in the case of the apparent restoration of some of these mechanisms (Section XII) it is those mechanisms which are last to disappear which return.

The sugars form an interesting class in that they appear to be associated with a particular portion or patch of the surface. This is indicated by results given in Sections VII to X. We consider that the best interpretation of the results is afforded by the assumption that on exposure of *B. coli* to ether, chloroform, benzene, toluene, phenol, etc., a film of the substance is formed upon certain patches of the cell surface, these portions being particularly associated with the activations of the sugars, and possibly being of a fatty or lipoidal nature. The formation of such a film would remove or diminish the accessibility of such highly polar compounds as the sugars and polyhydric alcohols and hence have the effect of eliminating the activating mechanisms of the latter. It is possible, too, that the actual solution of such substances as ether, etc., in the more fatty or lipoidal fractions of the surface will so alter the surface structure as entirely to destroy the groupings associated with the activations of the sugars.

It may be urged that the effect of ether, toluene, etc., is to dissolve off from the organism some material essential for the activation of the sugars, etc. This seems unlikely when it is considered

(1) that a small quantity of ether (benzene or toluene) or a short exposure is just as effective in removing all action with the sugars as a prolonged extraction with ether;

(2) that 1 % phenol has exactly the same effects as ether;

(3) that an activation for fructose appears to persist even after thorough extraction with ether.

Again, the action of ether, etc., cannot be interpreted as due to the alteration of cell permeability (though this doubtless occurs) when it is considered that although the treated cell is now presumably impermeable to glycol, glycerol and the sugars it must be still permeable to such relatively large molecules as methylene blue and α-glycerophosphoric acid.

The activations of succinic acid, formic acid, etc., appear to be associated with the less fatty or lipoidal fractions of the surface. This is indicated in Sections V and VII–X.

The experiment with "dialysed iron" (Section XI) indicates that a loose union with the organism, such as might be expected when oppositely charged particles mutually precipitate, has but little effect on the activations of the organism.

It is of interest that the mechanisms dealing with succinic acid and fumaric acid appear to be identical (see Tables VIII, IX, X). In every case when the succinic acid activation disappears so does that for fumaric acid. No selective action on the two mechanisms has been observed.

Summing up, it seems to us that the experimental results favour the views which have been put forward. No other interpretation appears satisfactorily to fit all the facts.

It may, perhaps, be emphasised once more that on these views a surface structure may induce a variety of reactions each differing in some way from the other so that the appearance of a large number of specific enzymes is obtained. The cell surface—or an intracellular surface—can be regarded as an assemblage of such enzymes but "enzymes which are inseparable because they are actually a function of the surface structure." As such structures become smaller in size the variety of reactions to which they give rise will become smaller and hence the specificity of its effects will apparently become greater until eventually a structure may be reached for which high specificity may be claimed. But there is no fundamental difference between such a highly specific structure and that of a cell surface (such as *B. coli*) which can accomplish over 50 activations.

Summary.

1. Resting *B. coli* was exposed to the following conditions and substances, and its activations, after exposure, were determined by the methylene blue technique.

(*a*) Changes of temperature from that of liquid air to 77°.

(*b*) Changes of H ion concentration from p_H 2·2 to p_H 11.

(*c*) Varying concentrations of sodium chloride at varying p_H and for different times.

(*d*) Varying concentrations of sodium nitrite at varying p_H and for different times.

(*e*) Methylene blue.

(*f*) Benzene, toluene, phenol, ether, chloroform, propyl alcohol and *cyclo*-hexanol.

(*g*) "Dialysed iron."

(*h*) Dilute solutions of $CuSO_4$ and $HgCl_2$ (preliminary experiments only).

2. Selectivity of destructive action on the activating mechanisms is exhibited by such exposures. A step by step degradation occurs. The order of sensitivity or of disappearance of the mechanisms is approximately the same in all cases.

3. The activations of the sugars appear to take place at the more fatty or lipoidal fractions of the surface; those of succinic acid, formic acid, etc., at the less lipoidal. It would seem that the activation of glucose as a H donator leads also to glycolysis.

4. Reversibility of action is demonstrated in the cases of propyl alcohol and *cyclo*-hexanol, and in the restoring effects of H_2S on mechanisms inactivated by copper or mercury. A restorative action by sodium hydrosulphite is described.

5. The experimental results are in favour of the view, previously propounded, that the activations of substrates are intimately associated with surface structure. The specificity of behaviour observed, as deduced from the selectivity of destructive action, is shown to be in agreement with this view of surface activation.

It is a great pleasure to express our sense of gratitude to and our appreciation of the interest taken in this work by Sir F. G. Hopkins. One of us (W. R. W.) is indebted to the Medical Research Council for a part time grant.

REFERENCES.

Böhm (1925). *Z. anorg. Chem.* **149**, 203.
Cole and Onslow (1916). *Lancet*, ii, 9.
Dixon (1926). *Biochem. J.* **20**, 714.
Hardy and Doubleday (1921). *Proc. Roy. Soc. Lond.* A. **100**, 550.
—— —— (1922). *Proc. Roy. Soc. Lond.* A, **101**, 487.
Myrbäck (1926). *Z. physiol. Chem.* **158**, 160.
Nicolai (1926). *Biochem. Z.* **179**, 86.
Quastel (1926). *Biochem. J.* **20**, 166.
Quastel and Whetham (1925, 1). *Biochem. J.* **19**, 520.
—— —— (1925, 2). *Biochem. J.* **19**, 645.
Quastel and Wooldridge (1925). *Biochem. J.* **19**, 652.

XXIII. CHEMISTRY OF THYROXINE.
III. CONSTITUTION AND SYNTHESIS OF THYROXINE.

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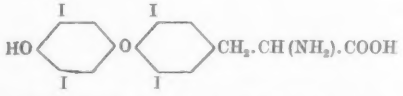
(*Received December 29th, 1926.*)

In recently published work it has been shown by one of us [Harington, 1926] that thyroxine is a tetraiodo-derivative of the *p*-hydroxyphenyl ether of tyrosine[1]; the orientation of the iodine atoms was left undetermined but it was suggested that they probably occupied the positions shown in the following formula:



The object of the present paper is in the first place to offer evidence in support of this suggestion, and in the second place to describe the synthesis of thyroxine itself[2].

The experimental and theoretical considerations leading to the above-mentioned supposition regarding the position of the iodine atoms were as follows.

(1) Thyroxine was fused with potash at a high temperature in the absence of oxygen, in the hope of obtaining identifiable degradation products in which iodine was replaced by hydroxyl. Although no pure substances could be isolated as the result of these experiments, the products of alkali fusion did, nevertheless, exhibit pyrogallol reactions, from which it appeared probable that one or both of the benzene rings of the thyroxine molecule had been converted into 3:4:5-trihydroxy-derivatives.

(2) Kendall and Osterberg [1919] have described a colour reaction with nitrous acid and ammonia as being characteristic of thyroxine; we have found that this colour reaction is given in general by benzene derivatives which contain two iodine atoms in the *ortho* positions to a hydroxyl (or amino-)

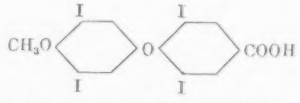
[1] I have since learned that Dr H. D. Dakin had come simultaneously to substantially the same conclusions as myself regarding the constitution of thyroxine; he had made the additional interesting observation that, on heating thyroxine with fuming hydriodic acid at 140°, tyrosine is formed. On hearing from Prof. Barger that I had communicated a paper on the subject to this *Journal*, Dr Dakin withdrew his paper, which was at that time in the hands of the Editor of the *Journal of Biological Chemistry*, from publication. C. R. H.

[2] The claim of Kendall and Osterberg [1919] to have synthesised thyroxine cannot be maintained in view of the facts that no experimental evidence has been offered in support of this claim, and, further, that the views of these authors regarding the constitution of thyroxine have been shown [Harington, 1926] to be wholly erroneous.

group; it is, for instance, given with intensity by diiodo-*p*-cresol, by 3:5-diiodo-4-hydroxybenzoic acid, and by 3:5-diiodotyrosine; this therefore made it likely that thyroxine contained a similar grouping.

(3) On general grounds one is almost bound to regard thyroxine as being derived from tyrosine, through the stage of 3:5-diiodotyrosine; assuming this to be the case, it is highly probable that thyroxine is formed in nature by the coupling of two molecules of diiodotyrosine with the loss of one side chain; such a reaction would lead to a compound of the suggested constitution.

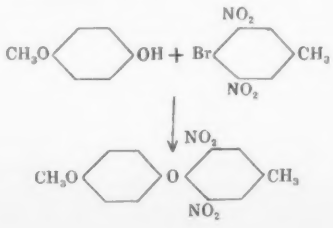
Admittedly these arguments cannot be regarded as convincing, but further evidence by methods of degradation appeared to be out of the question, and the solution of the problem could therefore only be obtained by synthesis. A certain simplification could indeed be effected by the methylation and subsequent oxidation of thyroxine, by a series of steps precisely similar to that described in the case of desiodothyroxine [Harington, 1926] leading to a tetraiodo-derivative of the acid $CH_3O.C_6H_4.O.C_6H_4.COOH$, and our first object was therefore the synthesis of the compound



and its comparison with the corresponding acid derived from thyroxine.

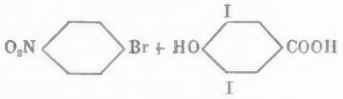
Early experiments on the direct iodination of desiodothyroxine itself and of other *p*-hydroxyphenyl ethers had indicated that these compounds would take up readily two atoms of iodine (though the products were very difficult to purify), but that the uptake of iodine ceased definitely at this point; no method was found for the direct introduction of more than two atoms of iodine. This being so, it was apparent that the desired tetra-iodophenyl ethers could only be obtained by the introduction of those two iodine atoms (or other groups which could be substituted by iodine) which were destined to occupy the 3:5 positons, *before* the phenyl ether synthesis was carried out. In other words, iodine atoms (or other replaceable groups) had to be present in the *ortho* positions either to the halogen atom or to the phenolic group which was to take part in the phenyl ether condensation.

In the first experiments in this direction quinol monomethyl ether was condensed with 3:5-dinitro-4-bromotoluene:



the resulting dinitrodiphenyl ether was successfully reduced to the diamine, but complete failure attended all attempts to replace the amino-groups in this compound by iodine.

This line of attack was therefore abandoned in favour of attempts to condense *p*-nitrohalogenbenzenes with 3:5-diiodo-4-hydroxybenzene derivatives, *e.g.* to condense *p*-nitrobromobenzene with 3:5-diiodo-4-hydroxybenzoic acid:

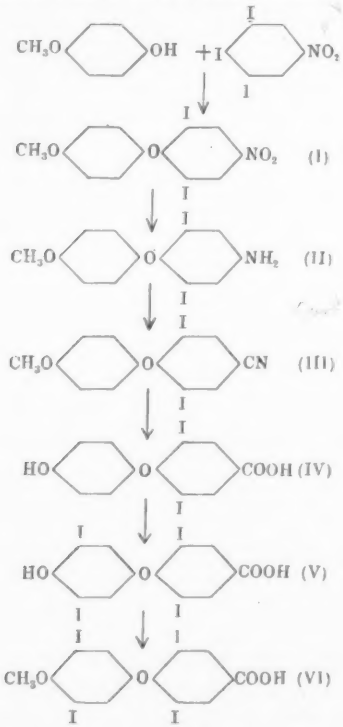


Much time was spent without success on experiments of this type. Mild treatment, such as warming the components in pyridine solution, or boiling in acetone with potassium carbonate, resulted only in the recovery of unchanged starting material, whilst more vigorous treatment, with the addition of copper bronze, caused the elimination of the relatively labile iodine atoms.

The solution of the difficulty was finally obtained in a different manner. As is well known, the presence of a nitro-group exerts a powerful mobilising effect on a halogen in the *para* position, a somewhat less marked effect on one in the *ortho* position, and no effect at all on one in the *meta* position. In accordance with this fact it was found that, of the three iodine atoms in 3:4:5-triiodonitrobenzene, that in the 4-position is so far preferentially mobilised that this substance can be condensed with a phenol to give a good yield of the 3:5-diiodo-4-phenoxynitrobenzene. Quinol monomethyl ether, therefore, was condensed with 3:4:5-triiodonitrobenzene to give 3:5-diiodo-4-(4′-methoxyphenoxy)nitro-benzene (I); this compound was reduced to the corresponding aniline (II) and the latter converted, by means of Sandmeyer's reaction, into the nitrile (III). This nitrile, on boiling with a mixture of hydriodic and acetic acids, underwent simultaneous hydrolysis and demethylation, yielding 3:5-diiodo-4-(4′-hydroxyphenoxy)benzoic acid (IV); on addition of iodine in potassium iodide to a solution of this acid in concentrated ammonia, iodine was rapidly taken up, the uptake ceasing abruptly at two molecules, and there was obtained a good yield of 3:5-diiodo-4′-(3′:5′-diiodo-4′-hydroxyphenoxy)benzoic acid (V); on methylation, this gave an acid (VI) which was in every respect identical with the acid obtained by the methylation and subsequent oxidation of thyroxine. This series of experiments, therefore, settled beyond doubt the question of the orientation of the iodine atoms in thyroxine, since it is certain that the last two iodine atoms, introduced into the acid (IV) in alkaline solution, must have entered the *ortho* positions to the free phenolic group.

As regards the actual synthesis of thyroxine, the most favourable starting point appeared to be 3:5-diiodo-4-(4′-methoxyphenoxy)benzaldehyde (VII) which could indeed be obtained in good yield from the nitrile (III) by the method of Stephen [1925]. It was obvious, however, that formidable diffi-

culties were to be expected, since the reduction which would be involved in the synthesis, from this aldehyde, of the corresponding α-aminopropionic acid would be likely to displace the iodine atoms; indeed, for this reason, an alkaline reduction was entirely out of the question, and prolonged heating with hydriodic acid and red phosphorus proved to be equally unsuccessful. The first success was obtained by condensing the aldehyde with hydantoin, boiling the product for one hour with hydriodic acid and a little phosphorus,



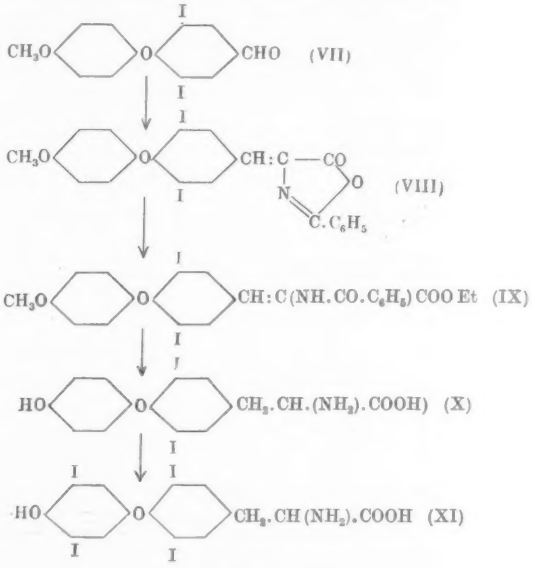
and hydrolysing the resulting compound with concentrated barium hydroxide; the intermediate products were, however, very insoluble and difficult to purify, and the yields were poor. Considerably better results were obtained as follows: the aldehyde (VII) was condensed with hippuric acid, and the resulting azlactone (VIII) converted into the corresponding α-benzoylamino-cinnamic ester (IX); this, on boiling for one hour with hydriodic acid and red phosphorus, gave a 25 % yield of

β-[3:5-diiodo-4-(4′-hydroxyphenoxy)]phenyl-α-aminopropionic acid (X).

On iodination in ammoniacal solution this compound yielded

β-[3:5-diiodo-4-(3′:5′-diiodo-4′-hydroxyphenoxy)]phenyl-α-aminopropionic acid (XI),

a substance which was entirely identical, in its chemical and physiological properties, with thyroxine isolated from the thyroid gland. By this synthesis, therefore, together with the confirmatory evidence offered by the synthesis of the iodine-containing degradation product described above, the constitution of thyroxine may be regarded as finally established.



Experimental.

A. *Degradation of thyroxine.*

Potash fusion of thyroxine. 0·5 g. thyroxine was mixed with 5 g. powdered potassium hydroxide and the mixture heated at 310° for 30 minutes in a silver tube through which a current of hydrogen was passing. After cooling, the melt was dissolved out with hydrochloric acid containing some sulphur dioxide. The product was not entirely soluble, a grey precipitate being obtained, which, as also the solution itself, darkened on exposure to air. On addition of excess of alkali the solution became immediately black. When the solution was carefully neutralised with ammonia and treated with dilute ferric chloride, colour reactions of the pyrogallol type were obtained. Variations of the duration and temperature of the fusion were tried, but in no case could any pure product be isolated.

Exhaustive methylation of thyroxine. 2·33 g. thyroxine were dissolved in 55 cc. methyl alcohol containing 1 % potassium hydroxide; 25 cc. of methyl iodide were added and the solution boiled under a reflux condenser; boiling was continued for 8 hours in all, additions of 10 cc. of 4 % methyl alcoholic

potassium hydroxide being made at half-hourly intervals, and two further quantities of 5 cc. of methyl iodide being added at 4 and 6 hours respectively; 100 cc. of water were then added and the methyl alcohol and excess of methyl iodide distilled off under reduced pressure; the colourless product which separated was filtered off and boiled with 50 cc. alcohol and 50 cc. 2 *N* potassium hydroxide until trimethylamine ceased to be evolved; the hot clear solution, on cooling, deposited 1·6 g. of the potassium salt of the unsaturated acid. The free acid, on crystallisation from glacial acetic acid, in which it was but little soluble, formed fine colourless needles which gave off iodine at 286° and melted indefinitely above 290°.

Analysis. 5·747 mg. gave 5·340 mg. CO_2; 0·80 mg. H_2O.
1·272 mg. required 7·73 cc. *N*/196 thiosulphate [Kendall, 1914].

	C	H	I
Found	25·3 %	1·5 %	65·75 %
Calc. for $C_{16}H_{10}O_4I_4$	24·8 %	1·3 %	65·5 %

Oxidation of unsaturated acid, $C_{16}H_{10}O_4I_4$. 0·81 g. of the potassium salt of the above acid was suspended in 35 cc. boiling water, and treated gradually with 1·5 *N* potassium permanganate; after the latter had been added in amount corresponding to 3 atoms of oxygen the oxidation ceased abruptly; apparently the aldehyde is so completely insoluble in water as to escape further oxidation under these conditions. The solution was treated with sulphur dioxide, and the amorphous precipitate, consisting entirely of neutral material, was filtered off, washed, dried and crystallised from glacial acetic acid; it formed sheaves and rosettes of fine colourless needles, M.P. 198°.

Analysis. 4·515 mg. gave 3·890 mg. CO_2; 0·51 mg. H_2O.
1·37 mg. gave 0·9489 mg. I [Kendall, 1914].

	C	H	I
Found	23·5 %	1·3 %	69·3 %
Calc. for $C_{14}H_8O_3I_4$	23·0 %	1·1 %	69·5 %

Oxidation of aldehyde, $C_{14}H_8O_3I_4$. 0·2 g. of the above aldehyde was dissolved in 4 cc. pure pyridine and the solution treated gradually, in the cold, with 0·6 cc. of 5 % potassium permanganate; after the latter had been entirely decolorised, the solution was diluted with water, treated with sulphur dioxide until colourless, made acid to Congo red with hydrochloric acid, and the white precipitate filtered off. The product was entirely soluble in a large amount of boiling dilute sodium carbonate, but, on cooling, the sodium salt separated almost completely in well-formed colourless needles. The free acid, crystallised from glacial acetic acid, formed a felt of fine colourless needles, M.P. 283°.

Analysis. 4·443 mg. gave 3·720 mg. CO_2; 0·53 mg. H_2O.
1·466 mg. gave 1·0019 mg. I [Kendall, 1914].

	C	H	I
Found	22·8 %	1·3 %	68·3 %
Calc. for $C_{14}H_8O_4I_4$	22·5 %	1·1 %	67·9 %

The *methyl ester*, prepared in the usual way, and recrystallised from acetic acid, formed flattened rhombic prisms, M.P. 233°; the *ethyl ester* formed long thin needles, M.P. 171·5°.

B. *Synthesis of acid degradation product,* $C_{14}H_8O_4I_4$ (VI).

3:4:5-Triiodonitrobenzene was most conveniently prepared from diiodo-*p*-nitraniline (obtained by the method of Willgerodt and Arnold [1901]) as follows: 75 g. diiodo-*p*-nitraniline were dissolved in 150 cc. concentrated sulphuric acid; the solution was cooled to 5° and vigorously stirred while finely powdered sodium nitrite (2·5 mols.) was slowly added; the stirring was continued for a further 2 hours, the temperature being maintained at 5–10° throughout. The mixture was then poured on to ice and treated with a concentrated aqueous solution of potassium iodide (5 mols.). After the first vigorous reaction, the solution was warmed for some time on the water-bath; it was then cooled, free iodine was removed with bisulphite, and the granular precipitate filtered off, washed with water, alcohol and ether, and dried. The most convenient purification was effected by distillation *in vacuo*, which gave a clean product, M.P. 165°, in a yield of 75–80 % of the theoretical. The M.P. of this compound is given by Willgerodt and Arnold [1901] as 105°; in a later paper by Repossi [1916] the figure given is 167°[1].

3:5-*Diiodo*-4-(4′-*methoxyphenoxy*)*nitrobenzene* (I). This compound was prepared by boiling a solution of quinol monomethyl ether and 3:4:5-triiodonitrobenzene in methylethylketone with dry potassium carbonate. If molecular proportions were employed, the product contained a good deal of unchanged triiodonitrobenzene which is difficult to remove; it was found better, therefore, to use twice the theoretical amount of the phenol and potassium carbonate. It is further essential for the success of this reaction that the potassium carbonate should be freshly dried and finely powdered. 50 g. 3:4:5-triiodonitrobenzene and 25 g. quinol monomethyl ether were dissolved in 250 cc. boiling methylethylketone; 30 g. finely powdered anhydrous potassium carbonate were added and the solution boiled under a reflux condenser for 16 hours; it was then poured into water containing a slight excess of acetic acid, and the methylethylketone and excess of quinol monomethyl ether were removed by steam distillation; after cooling, the precipitate was filtered off, and crystallised, first from glacial acetic acid, and then from methylethylketone; there resulted 33–35 g. of a product which was practically pure, *i.e.* a yield of about 67 %. For complete purification the compound was distilled *in vacuo* (B.P. 260°/3 mm.) and crystallised from methylethylketone. It formed yellow elongated prisms, M.P. 144°. It is insoluble in water, slightly soluble in alcohol and ether, much more readily so in chloroform and acetic acid.

Analysis. 0·2257 g. gave 0·2593 g. CO_2; 0·0474 g. H_2O.
0·1581 g. gave 0·1502 g. AgI.
20·1 mg. gave 0·523 mg. N (modified micro-Kjeldahl).

	C	H	N	I
Found	31·3 %	2·3 %	2·6 %	51·3 %
Calc. for $C_{13}H_9O_4NI_2$	31·2 %	1·8 %	2·8 %	51·1 %

[1] This compound has also been recently prepared in a similar manner by Kalb, Schmeizer, Zellner and Berthold (*Ber. deutsch. chem. Ges.* 1926, **59**, 1860. [C. H. R. February 2, 1927.]

3:5-*Diiodo*-4-(4′-*methoxyphenoxy*)*aniline* (II). 25 g. of the above nitro-compound were dissolved in 125 cc. hot glacial acetic acid; 37·5 g. powdered stannous chloride were added, and a rapid stream of dry hydrogen chloride passed into the solution, which was kept hot on the water-bath. After a time the stannichloride of the base began to separate; when the precipitate no longer increased in amount and the solution was saturated with hydrochloric acid (about 45 minutes), it was allowed to cool; the crystalline stannichloride was then filtered off, washed with glacial acetic acid, decomposed as rapidly as possible by grinding with 250 cc. of 40 % sodium hydroxide, and the base immediately extracted with ether. If the operation had been properly conducted, the ethereal solution was but little coloured, in which case it was used, after drying, for the direct preparation of the hydrochloride of the base which was required for the next reaction. The free base was obtained pure by evaporating the dried ethereal solution and recrystallising the residue, first from a very small amount of benzene, and then from ligroin. It formed colourless narrow rhombic prisms, M.P. 121–122°. It was very slightly soluble in hot water, but easily soluble in most organic solvents except light petroleum.

Analysis. 0·1782 g. gave 0·2204 g. CO_2; 0·0426 g. H_2O.
0·1629 g. gave 0·1634 g. AgI.
34·2 mg. gave 1·024 mg. N (micro-Kjeldahl).

	C	H	N	I
Found	33·7 %	2·6 %	2·7 %	54·2 %
Calc. for $C_{13}H_{11}O_2NI_2$	33·4 %	2·4 %	3·0 %	54·4 %

The *hydrochloride* formed colourless needles, sparingly soluble in water, M.P. 216° after preliminary sintering; the *sulphate* formed colourless needles, with similar solubilities, M.P. 201° after sintering. The base and its salts have a distinctly irritating effect on the skin, which necessitates considerable care in handling them.

3:5-*Diiodo*-4-(4′-*methoxyphenoxy*)*benzonitrile* (III). The diazotisation of the base just described offered, at first, considerable difficulties, since, in the presence of excess of mineral acid (especially of sulphuric acid) complex coloured condensation products were readily formed. The reaction can however be conveniently accomplished by treatment of a suspension of the dry hydrochloride in glacial acetic acid with amyl nitrite. 30 g. of the hydrochloride, prepared by saturating the ethereal solution of the base with dry hydrogen chloride, were suspended in 300 cc. of glacial acetic acid; the suspension was vigorously stirred and kept at 15–20° while amyl nitrite was gradually added in slight excess; the hydrochloride rapidly disappeared and there was obtained a clear orange-coloured solution of the diazonium salt. For the preparation of the nitrile, this solution was poured, with stirring, into a hot solution prepared by adding 170 g. potassium cyanide in 300 cc. water to 150 g. copper sulphate in 600 cc. water. (It was found to be necessary to use the very large excess of cuprous cyanide here indicated, since otherwise the diazonium group was very readily exchanged for hydrogen.) The precipitate was filtered off, washed with water, and boiled out with benzene; the

benzene solution was filtered and dried over calcium chloride. The benzene was then evaporated and the residue distilled under reduced pressure; the dark-coloured, but crystalline, distillate was recrystallised from methylethylketone, and 13–15 g. of a substance forming colourless prisms, M.P. 167–169°, were obtained.

Analysis. 0·2145 g. gave 0·2760 g. CO_2; 0·0382 g. H_2O.
0·1650 g. gave 0·1625 g. AgI.
20·0 mg. gave 0·574 mg. N (micro-Kjeldahl).

	C	H	N	I
Found	35·1 %	2·0 %	2·9 %	53·2 %
Calc. for $C_{14}H_9O_2NI_2$	35·2 %	1·9 %	2·9 %	53·2 %

3:5-*Diiodo*-4-(4′-*hydroxyphenoxy)benzoic acid* (IV). 5 g. of the nitrile were dissolved in 40 cc. glacial acetic acid, and to the boiling solution, contained in a flask under a reflux condenser, were slowly added 50 cc. hydriodic acid (Sp. G. 1·7) so as to cause as little precipitation of the nitrile as possible; the whole was then boiled for 2 hours. On cooling and diluting with water a partly crystalline precipitate separated, which was filtered off and dissolved in warm dilute ammonia; the filtered ammoniacal solution was acidified and the acid filtered off, dried and crystallised from 50 % alcohol; it formed long colourless needles, M.P. 252–254°. The yield was 80 % of the theoretical.

Analysis. 0·1683 g. gave 0·2004 g. CO_2; 0·0275 g. H_2O.
0·1626 g. gave 0·1592 g.AgI.

	C	H	I
Found	32·5 %	1·8 %	52·8 %
Calc. for $C_{13}H_8O_4I_2$	32·3 %	1·7 %	52·7 %

During the above-described hydrolysis there was always formed a certain amount of a compound containing no nitrogen, and exhibiting phenolic but no acidic properties; this was not investigated further.

3:5-*Diiodo*-4-(3′:5′-*diiodo*-4′-*hydroxyphenoxy)benzoic acid* (V). Attempts to iodinate the diiodo-acid, by the usual method for phenols, in solution in sodium or potassium hydroxide, met with no good result; the reaction proceeded smoothly, however, in ammoniacal solution by the method of Datta and Prosad [1917]. 0·964 g. of the diiodo-acid was dissolved in 8 cc. of concentrated ammonium hydroxide (Sp. G. 0·880); to this solution was added, drop by drop, with constant shaking, a normal solution of iodine in potassium iodide; as each drop was added the black precipitate of nitrogen iodide disappeared instantaneously, on shaking, until almost the theoretical 2 mols. of iodine had been added; with further addition of iodine, the black precipitate disappeared more slowly, until, when the theoretical amount (or one or two drops in excess) had been added, the dark colour became permanent, indicating the presence of nitrogen iodide in excess; the end-point of the reaction was almost as sharply defined as that of a titration. After about 75 % of the iodine had been added a crystalline ammonium salt began to separate and increased in amount until the end of the reaction; addition of saturated ammonium chloride solution precipitated still more of this salt, the total amount of which was 93 % of the theoretical for the ammonium salt of the

tetra-iodo-acid. The free acid, crystallised from glacial acetic acid, formed bunches of colourless needles, M.P. 255°, with decomposition and evolution of iodine.

Analysis. 0·1045 g. gave 0·1325 g. AgI.

Found I 68·5 % Calc. for $C_{13}H_6O_4I_4$ I 69·2 %

3:5-*Diiodo*-4-(3′:5′-*diiodo*-4′-*methoxyphenoxy*)*benzoic acid* (VI). On methylation with dimethyl sulphate and potassium hydroxide in the usual manner, the above-described tetra-iodohydroxy-acid yielded the methoxy-acid, which, when crystallised from glacial acetic acid, formed a felt of colourless needles, M.P. 286°.

Analysis. 1·31 mg. required 8·4 cc. *N*/200 sodium thiosulphate [Kendall, 1914].

Found I 68·0 % Calc. for $C_{14}H_8O_4I_4$ I 67·9 %

The *sodium salt* of the acid was very sparingly soluble in water and crystallised in fine needles; the *methyl ester*, crystallised from acetic acid, formed flattened rhombic prisms, M.P. 233·5°; the *ethyl ester* similarly gave long fine needles, M.P. 172·5°. The results of comparison of this acid and its derivatives with the corresponding compounds obtained, as described above, by degradation of thyroxine are summarised in Table I; they leave no doubt as to the identity of the synthetic acid with that obtained from natural sources.

Table I.

	Crystalline form	M.P.	Mixed M.P.
Synthetic acid	Felted needles	286°	283–4°
Natural acid	,,	283°	
Synthetic methyl ester	Flattened rhombic prisms	233·5°	233°
Natural ,,	,, ,,	233°	
Synthetic ethyl ester	Long needles	172·5°	172·5°
Natural ,,	,,	171·5°	

C. *Synthesis of thyroxine.*

3:5-*Diiodo*-4-(4′-*methoxyphenoxy*)*benzaldehyde* (VII). Our first attempts to prepare this aldehyde from the corresponding nitrile, using the conditions recommended by Stephen [1925], *i.e.* 1·5 mols. of stannous chloride and a period of 2 hours' standing, resulted in minute yields only. Much better results were obtained by increasing the proportion of stannous chloride to 4 mols., and allowing the mixture to stand overnight, or even longer. 5 g. of 3:5-diiodo-4-(4′-methoxyphenoxy)benzonitrile (see above) were dissolved in 35 cc. chloroform and added, with vigorous shaking, to a solution of 12 g. anhydrous stannous chloride in 60 cc. dry ether saturated with hydrogen chloride; after standing for at least 16 hours the yellow precipitate was filtered off, washed with ether and warmed with water containing a little hydrochloric acid; at the boiling point the hydrolysis was almost instantaneous, the yellow stannichloride being replaced by a colourless flocculent precipitate of aldehyde; this was filtered off, washed with dilute hydrochloric acid and then with water, dried, and crystallised from glacial acetic acid; it formed colourless prisms, M.P. 121°. The yield varied from 70 % to 100 % of the theoretical. The

aldehyde was practically insoluble in water, but fairly soluble in all organic solvents except light petroleum.

Analysis. 0·1643 g. gave 0·2131 g. CO_2; 0·0324 g. H_2O.
0·1479 g. gave 0·1447 g. AgI.

	C	H	I
Found	35·3 %	2·2 %	52·9 %
Calc. for $C_{14}H_{10}O_3I_2$	35·0 %	2·1 %	52·9 %

The *phenylhydrazone* formed long yellow needles, M.P. 175–176°.

Azlactone from above aldehyde and hippuric acid (VIII). An intimate mixture of 5 g. of the aldehyde, 5 g. freshly fused sodium acetate, and 1·9 g. hippuric acid was treated with 15 cc. acetic anhydride and heated on the water-bath for 15 minutes; the yellow reaction mixture was ground up with water, filtered off, well washed and dried; the yield was 6 g. of an almost pure product. Crystallised from glacial acetic acid, it formed long, bright yellow needles, M.P. 211°.

Analysis. 15·5 mg. gave 0·303 mg. N (micro-Kjeldahl).
0·1539 g. gave 0·1174 g. AgI.

	N	I
Found	2·0 %	41·2 %
Calc. for $C_{23}H_{15}O_4NI_2$	2·2 %	40·9 %

α-Benzoylamino-3:5-diiodo-4-(4′-methoxyphenoxy)cinnamic ester (IX). The azlactone was warmed on the water-bath for 45–60 minutes with 75 parts of absolute alcohol containing 10 % sulphuric acid; the clear and almost colourless solution was poured into water and the precipitate collected, washed and dried. The yield was practically quantitative; the compound crystallised from glacial acetic acid, or from methylethylketone, in colourless needles, which softened at 195° and melted at 203°.

Analysis. 13·9 mg. gave 0·267 mg. N (micro-Kjeldahl).
0·1482 g. gave 0·1051 g. AgI.

	N	I
Found	1·9 %	38·3 %
Calc. for $C_{25}H_{21}O_5NI_2$	2·1 %	38·0 %

β-[3:5-Diiodo-4-(4′-hydroxyphenoxy)phenyl]-α-aminopropionic acid (X). 5 g. of the benzoylaminocinnamic ester were boiled under a reflux for one hour with 25 cc. hydriodic acid (Sp. G. 1·7) and 3 g. red phosphorus; the solution was filtered hot through asbestos and evaporated to dryness *in vacuo*; some water was added and the evaporation repeated; the residue was dissolved in warm water with the aid of a little hydrochloric acid, and filtered from some tar. In order to avoid separation of the somewhat insoluble hydrochloride of the amino-acid it was necessary not to cool the acid filtrate more than could be helped; as soon as it was cool enough it was shaken out once with ether, which removed the whole of the coloured impurities; the aqueous solution was then brought to the boil and treated with saturated sodium acetate until no longer acid to Congo red; the amino-acid immediately separated in beautiful silvery platelets, which, after cooling, were filtered off, washed with water, alcohol and ether, and dried. The yield was 1 g. of the

pure substance, or 25 % of the theoretical. The compound gave the ninhydrin reaction, but was too insoluble to permit of a satisfactory determination of amino-nitrogen by the Van Slyke method. It melted at 245–6°.

Analysis. 18·1 mg. gave 0·475 mg. N (micro-Kjeldahl).
0·1270 g. gave 0·1118 g. AgI.

	N	I
Found	2·6 %	47·6 %
Calc. for $C_{15}H_{13}O_4NI_2$	2·7 %	48·3 %

β-[3:5-Diiodo-4-(3′:5′-diiodo-4′-hydroxyphenoxy)phenyl]-α-aminopropionic acid—Thyroxine (XI). 0·525 g. of the diiodoamino-acid was dissolved in 11–12 cc. concentrated ammonium hydroxide (Sp. G. 0·880) and treated, drop by drop, with constant shaking, with *N* iodine in potassium iodide (2 mols.); towards the end of the reaction, the uptake of iodine, which until then had been practically instantaneous, showed a distinct slackening; during the later stages of the reaction a crystalline ammonium salt had begun to separate; after all the iodine had been added, the solution was allowed to stand for some time, and the ammonium salt was then separated on the centrifuge; the salt was dissolved in alcohol with the aid of a little dilute sodium hydroxide, the solution was filtered, boiled and acidified with acetic acid; there separated immediately a colourless crystalline precipitate, which exhibited the very characteristic crystalline form of thyroxine (rosettes and curiously shaped sheaves of colourless needles). This fraction amounted to 0·33 g. A further amount was obtained from the mother-liquor of the ammonium salt as follows: the solution was diluted and boiled until the ammonia was removed; the partly crystalline precipitate which had separated was filtered off and dissolved, so far as possible, in boiling 0·5 % sodium carbonate; the filtered solution, on cooling, deposited a heavy white micro-crystalline precipitate, with the typical appearance of the sodium salt of thyroxine; this was separated on the centrifuge and further treated as described above for the ammonium salt; in this way 0·07 g. of somewhat less well-defined crystalline material was obtained, the total yield, therefore, being 0·4 g., or a little over 50 % of the theoretical. The substance melted at 231° with decomposition; when mixed with a sample of natural thyroxine of M.P. 228°, the mixture melted at 228°; it gave with intensity the colour reaction with nitrous acid and ammonia which is given by thyroxine; further, the solubilities of the natural and synthetic products and of their sodium salts were entirely similar.

Analysis. 24·6 mg. gave 0·447 mg. N (micro-Kjeldahl).
0·99 mg. required 6·08 cc. *N*/200 sodium thiosulphate [Kendall, 1914].

	N	I
Found	1·82 %	65·1 %
Calc. for $C_{15}H_{11}O_4NI_4$	1·82 %	65·3 %

In so far, then, as the matter is susceptible of decision by chemical methods, the identity of the synthetic product with natural thyroxine may be regarded as established. For the physiological confirmation we are indebted to Prof.

D. Murray Lyon of the University of Edinburgh, a note by whom, on the physiological test, is appended.

We have had the advantage of repeated discussion of the problem with Prof. R. Robinson, F.R.S., and we desire to acknowledge the assistance of Dr C. P. Stewart and of Mr W. McCartney in some of the earliest and latest experiments respectively.

Note on Physiological Test of Synthetic Thyroxine.

By D. MURRAY LYON.

Department of Therapeutics, University of Edinburgh.

Case 1. Mrs A. S. Age 61. Housewife.

Known to be myxoedematous in 1921, probably longer. Admitted to hospital in October 1922 showing typical picture of that condition. The patient improved for a time under thyroid extract, but extract of anterior lobe of pituitary did not prevent relapse. Thyroid medication was not continued after discharge from hospital (January 1923).

November 24th, 1926. Third admission to hospital. The patient has taken no care of herself and has never had thyroid extract outside of hospital. She now shows the classical signs of the disease, is dull mentally, is overweight, and has the typical facies and myxoedematous deposits and the harsh dry skin.

The B.M.R. was practically the same as on her previous admission. On November 27th 5 mg. synthetic thyroxine was dissolved in a drop of normal NaOH and made up to 1·5 cc. with distilled water, and was given intravenously. Next day the patient complained of headache and palpitation. By November 29th, two days after the first injection, the B.M.R. had risen from $-32\,\%$ to $-17\,\%$. On the 30th the patient herself remarked on her improved condition. She was much brighter and more cheerful, her swellings were diminishing, and wrinkling was obvious on the backs of the hands and under the eyes. Two further doses of 4 and 5 mg. given on November 29th and December 2nd raised the B.M.R. to $-6\,\%$ by December 4th. During this period both pulse rate and temperature rose somewhat. A fourth dose given on December 6th seems to have had little effect, as B.M.R., pulse rate and temperature have all declined from about this date. By the 18th the patient had practically returned to her previous condition with B.M.R. $-34\,\%$. The skin had become harsh again and the mental condition much more dull.

The close parallelism between B.M.R. and pulse rate will be noticed in Fig. 1.

Case 2. Mrs M. M. Age 35. Housewife.

Normal health until 1916 when she was noticed to be becoming dull mentally and disinclined for effort. Definitely diagnosed myxoedema in 1920. For next few years took thyroid tablets irregularly.

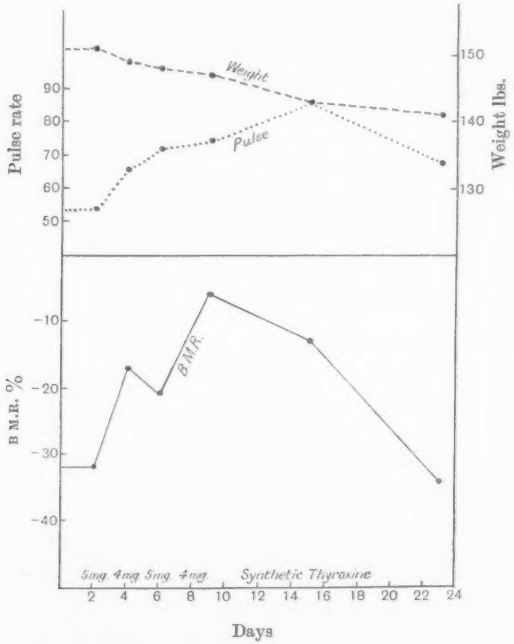


Fig. 1.

January 1926 admitted to Royal Infirmary, Edinburgh, on account of lack of energy and great increase in her swellings. Picture typically that of advanced myxoedema, with a B.M.R. of − 46 %. Remained in hospital from January 5th to March 29th while the dosage of thyroid extract was adjusted. Twelve grains a day proved to be too much and the B.M.R. rose to + 25 %. She was finally discharged on 6 grains a day with a B.M.R. of + 4 %.

Re-admitted November 24th, 1926, not having had any thyroid extract for some months. B.M.R. again − 45 %, the patient being markedly myxoedematous. Three intravenous injections of synthetic thyroxine (5, 4 and 5 mg.) were given on November 27th and 29th, and December 2nd. These produced a marked rise in the B.M.R. to + 3 %, the temperature and the pulse rate rising simultaneously. A fall in weight took place during the same period—partly due to great loss of fluid from the system. A very considerable diuresis followed the first two doses of thyroxine. Each injection also gave rise to an

attack of diarrhoea. As the result of the thyroxine the patient improved remarkably for a time, but after it was discontinued, she slipped back to her former state. In this patient a greater and more lasting effect was produced than in Case 1 (see Fig. 2).

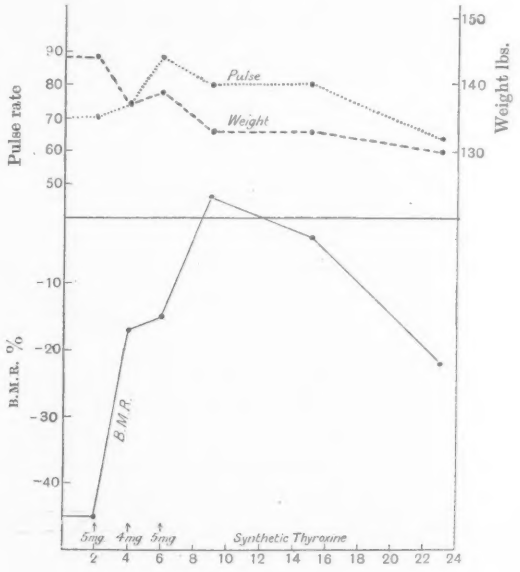


Fig. 2.

The results obtained in the above two cases are shown in Figs. 1 and 2 respectively. The effect of the synthetic thyroxine in raising the basal metabolic rate of these two patients is quantitatively similar to that reported by Boothby and Sandiford [1924] for natural thyroxine.

REFERENCES.

Boothby and Sandiford (1924). *Physiol. Reviews*, **4**, 69.
Datta and Prosad (1917). *J. Amer. Chem. Soc.* **39**, 441.
Harington (1926). *Biochem. J.* **20**, 300.
Kendall (1914). *J. Biol. Chem.* **19**, 251.
Kendall and Osterberg (1919). *J. Biol. Chem.* **40**, 265.
Repossi (1916). *Z. Kryst.* **55**, 281.
Stephen (1925). *J. Chem. Soc.* **127**, 1874.
Willgerodt and Arnold (1901). *Ber. deutsch. chem. Ges.* **34**, 3344.

XXIV. ECHINOCHROME.

By ROBERT KEITH CANNAN.

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(*Received December 29th, 1926.*)

Echinochrome is the name which was given by MacMunn [1885, 1889] to a pigment contained in the cells of the perivisceral fluid of *Strongylocentrotus lividus*, *Amphidotus cordatus*, *E. sphaera* and *E. esculentus*. On the grounds of an observed change in the spectrum under the action of strong reducing agents, MacMunn concluded that the pigment was an oxygen carrier. Griffiths [1892] confirmed this opinion but stated that the oxygen was much more firmly held than in oxyhaemoglobin, being removable only by the reducing action of the living cells. Decomposition of the pigment with boiling mineral acids was reported to yield a haemochromogen and a porphyrin. From the elaeocytes of the perivisceral fluid of the deeply coloured sea urchin, *Arbacia punctulata*, McLendon [1912] separated a pigment in a partially purified state, which he found to be spectroscopically identical with the pigment of MacMunn and the name echinochrome was, therefore, extended to it. McLendon found that the red colour of the eggs and of the test of *Arbacia* was due also to the same substance. His preparations contained no significant quantity of iron and he demonstrated that solutions of the pigment which had been submitted to a vacuum did not subsequently absorb any appreciable quantity of oxygen from the air. He consequently questioned the respiratory function of echinochrome.

During a visit to Woods Hole, the writer made the casual observation that the red colour of the aqueous extract of the elaeocytes of *Arbacia* was immediately discharged by sodium hydrosulphite and returned rapidly on shaking with air. In the absence of oxygen, the reduced substance was immediately re-oxidised by mild oxidising agents. The experiment was repeated titrimetrically and the electrode potentials observed. Substantial agreement between the titration curve traced during the reduction of the pigment with standard sodium hydrosulphite and during its re-oxidation with potassium ferricyanide, together with the rapid attainment and constancy of the electrode potentials, indicated that a reversible electrochemical system was under study. A small amount of the crude pigment was prepared and brought back to this country.

Experimental.

Following, in general, the method of McLendon, the echinochrome was extracted from the test of *Arbacia* with acetone. The extract was evaporated to dryness in a vacuum and the residue extracted with alcohol. Addition of ammonia to the alcoholic solution precipitated the pigment, which was then dissolved in faintly acidified water and the solution extracted with ether. This preparation was far from pure and, as its mass was small, it was, after further fractionation on similar principles, made up to a standard volume in water and this stock solution, preserved on ice, was used for the whole of the work to be reported.

The electrode studies took the usual form of determining the oxidation-reduction titration curve at a series of known [H·]; the detail has been described by Cannan [1926]. Gold plated platinum electrodes were employed, the standard half cell being a saturated calomel electrode. 10 cc. of the echinochrome solution were added to 50 cc. of a buffer [Clark, 1923] of known p_H and titrated in pure nitrogen with a standard deaerated solution of the oxidising agent similarly diluted with the buffer. The p_H of each of these mixtures was assumed to be identical with that of the buffer solution diluted to the same extent with water. The latter was determined with the hydrogen electrode. The curves traced by using benzoquinone or ferricyanide as oxidising agents were identical within the experimental error. Titration of the oxidised pigment with sodium hydrosulphite was vitiated by slow persistent drifts of potential rendering accurate observations impossible. Within the accuracy of observation, however, agreement with the oxidation titration curves was obtained.

The affinity of the reduced pigment for oxygen was so great that much care was necessary to preserve solutions of it from re-oxidation by air. For this reason it was found more certain to repeat the whole titration curve at a series of [H·] than to make a series of observations of the potentials of a standard mixture of oxidised and reduced forms added to various buffer mixtures.

Analysis of the experimental curves showed that they were adequately expressed by the electrode equation

$$E_h = E_o' - 0{\cdot}03006 \log \frac{[S_r]}{[S_o]}$$

where E_h is the observed potential compared with that of the normal hydrogen electrode, and $[S_r]$ and $[S_o]$ are the respective concentrations of the oxidised and reduced forms computed from the titre and the end-point determined graphically. E_o' is a constant for a given [H·]. The value of the numerical factor indicates that the reversible electrode reaction is one involving two equivalents of hydrogen, thus placing echinochrome in the same group as the majority of organic oxidation-reduction systems.

Table I records the abbreviated data of a typical titration curve and indicates that the observations were within the accuracy of the experimental

technique. From the titration curves at other [H·] the values of E_0' have been deduced and are given in Table II. These serve to define the system over the p_H range 2 to 7·7 with some certainty.

Table I. *Titration of reduced echinochrome with M/500 potassium ferricyanide.*

p_H = 3·97 (10 cc. echinochrome solution added to 50 cc. *M*/20 acid potassium phthalate). Temp. 30°. $E_0' = -0·0389$.

Ferricyanide cc.	Oxidation %	E_h (volts) Found	Calc.	Deviation
0·40	2·90	−0·0852	−0·0847	−0·0005
1·00	7·25	0·0722	0·0711	−0·0011
1·60	11·60	0·0659	0·0654	−0·0005
2·20	15·94	0·0604	0·0606	+0·0002
2·80	20·29	0·0567	0·0568	+0·0001
3·40	24·63	0·0534	0·0535	+0·0001
4·60	33·34	0·0481	0·0479	−0·0002
5·80	42·03	0·0429	0·0431	+0·0002
7·00	50·72	0·0387	0·0385	−0·0002
8·20	59·41	0·0339	0·0339	—
9·40	68·11	0·0288	0·0290	+0·0002
10·60	76·81	0·0231	0·0233	+0·0002
11·80	85·51	0·0155	0·0158	+0·0003
12·40	89·84	0·0111	0·0105	−0·0006
13·00	94·19	−0·0034	−0·0026	−0·0008
13·40	97·09	+0·0053	+0·0069	−0·0016
13·80	end point			

The data may be adequately expressed by the equation

$$E_0' = E_0 + 0·0601 \log [H^·],$$

i.e. the value of E_0' decreases by 0·0601 v. per unit increase in p_H. The deviations from the "0·06 slope" at p_H 8·78 and 9·76 may indicate critical acid-base dissociations with ionisation constants in this region. On the other hand, they may be due to the employment of borate buffers, since experience has shown that the presence of borate sometimes leads to anomalous potentials. Neglecting, therefore, these two results, the mean value of E_0 is + 0·1995.

Table II. *Relation of* E_0' *to* p_H.

p_H	E_0'	0·0601 p_H	E_0
2·21	+0·0668	−0·1328	+0·1996
3·02	+0·0176	0·1815	0·1991
3·97	−0·0389	0·2386	0·1997
4·97	−0·0992	0·2987	0·1995
6·01	−0·1614	0·3612	0·1998
6·75	−0·2056	0·4057	0·2001
7·16	−0·2308	0·4303	0·1995
7·69	−0·2632	0·4622	0·1990
8·78	−0·3220	0·5277	(0·2057)
9·76	−0·3774	0·5866	(0·2092)
		Mean	+0·1995

E_0 from titration of fresh filtered aqueous extract of elaeocytes (Woods Hole):

6·74	−0·201	−0·405	+0·204

From a crude experiment at Woods Hole on a fresh aqueous extract of the cells of the perivisceral fluid a value of + 0·204 for E_0 was obtained and

another experiment on an extract of the eggs was in close agreement. These results suggest that the pigment had been extracted in an unmodified condition and permit the conclusion that the system which has been defined above actually exists within the cells of *Arbacia*.

Attempts to demonstrate the formation of a compound of reduced echinochrome with molecular oxygen were negative. Moreover, the oxidant produced from reduced echinochrome by oxygen was identical with that formed by mild oxidising agents in the absence of oxygen. This follows from the fact that the experimental titration curves were duplicated by addition to reduced echinochrome of increasing amounts of the pigment formed by atmospheric oxygen.

An attempt was made to confirm these results on the pigment contained in *E. esculentus*. Very little pigment is, however, present and the amount extracted was insufficient for extended study. It was found, that the pigment did represent the oxidant of a reversible oxidation-reduction system but preliminary determinations of the electrode potentials suggested that these were distinctly positive to those of *Arbacia* echinochrome. Moreover, the solubilities of the two pigments differed. These qualitative observations are of interest because it would appear that *E. esculentus* holds its pigment in the partially reduced state, since the perivisceral fluid is almost colourless but rapidly turns red when removed from the animal. In *Arbacia* the echinochrome is in the oxidised state and I know of no observation of the spontaneous decolorisation of the cells *in vivo*. It would be of interest to know if the cells of *Arbacia* can effect a significant reduction of their pigment anaerobically—bacteria and yeast will slowly reduce it in the absence of oxygen—but the author has no longer access to the biological material.

The rate of oxidation of reduced echinochrome by atmospheric oxygen was too great to be measured by any simple means.

Discussion.

If any respiratory rôle is to be attributed to echinochrome it is clear that it must be that of an "activator" rather than of a "carrier" of oxygen. It forms no dissociable compound with molecular oxygen. It is of interest to consider this possibility briefly in the light of the quantitative data presented since it represents a concrete case of a type of respiratory catalyst frequently postulated. At the same time it must be acknowledged that the function suggested for echinochrome is at present without physiological proof.

It is probable that within living cells there exists a dynamic equilibrium—an oxidation-reduction poise—between the inherent reducing activity of the cells and the supply of active oxygen [Cannan, Cohen and Clark, 1926]. A characteristic reduction potential may be attributed to the cell, and the state of oxidation of the echinochrome present will be conditioned by this. If the assumption be made that the pigment is the effective oxygen activator then the rate of oxygen consumption of the cell will be determined by the con-

centration of reduced pigment in equilibrium—since its oxidation by molecular oxygen is very rapid. It is improbable that the cells can, aerobically, achieve a reduction intensity (corresponding to r_H 7) sufficient to reduce a measurable fraction of the total pigment present but it is clear from the electrode equation that at reduction intensities much less than this there remains a concentration of reduced pigment which, whilst analytically insignificant, may be a sufficient molecular concentration to insure a measurable oxygen consumption.

One further implication is of interest. It is well known that fertilisation of the egg of the sea urchin is followed by an enormous increase in the oxygen consumption. J. and D. Needham [1926] failed to observe a coincident increase in the reduction potential of the egg by their method of the micro-injection of indicators, although such an increase was to be expected. Now a fall in r_H of only one unit (an increase in reduction potential of 60 millivolts) would lead to a tenfold increase in the concentration of reduced echinochrome present. The method of injection of indicators was, in the present case, incapable of observing any change in r_H less than two or three units so that it was possible for a change to have occurred corresponding to an increase of several hundredfold in the concentration of reduced pigment, and, therefore, of oxygen consumption. It will be seen that in such a hypothetical system there is wide metabolic latitude although the system is well poised. Its investigation, however, requires exact information of the rate of oxidation of reduced echinochrome and of the reduction potential of the cell under the experimental conditions.

It has frequently been recorded that in the eggs of *Arbacia* fertilisation is followed by a migration of the chromatophores to the periphery of the cell. This might be considered to be an accessory, or alternative, means for increasing the concentration of reduced pigment in effective contact with oxygen. On the other hand, though this is improbable, the supposed migration may mask an actual reduction of the echinochrome in the interior of the cell owing to an enhanced reduction intensity following fertilisation.

One final qualification must be made. We have no present means of deciding to what extent the homogeneous equilibria represented by electrode potentials are modified in a heterogeneous system such as the living cell. This is particularly cogent in the present case where the pigment exists in granules microscopically distinguishable as a separate phase. The physiological application of electrometric data must be made with caution.

Summary.

1. Echinochrome has been separated from the eggs, perivisceral fluid and test of *Arbacia punctulata*. It does not form a dissociable compound with oxygen but is the oxidant of a reversible oxidation-reduction system.

2. The electrode potentials of the system have been measured over the p_H range 2·2–9·76. The normal electrode potential is + 0·1995.

3. The alleged respiratory function of echinochrome is discussed.

For the hospitality of the Marine Biological Laboratories of Woods Hole and of Plymouth the author is indebted to Dr F. Lillie and to Dr E. G. Allen respectively.

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REFERENCES.

Cannan (1926). *Biochem. J.* **20**, 927.
Cannan, Cohen and Clark (1926). *U.S. Public Health Rep. Suppl.* 55.
Clark (1923). The Concentration of Hydrogen Ions (2nd edition).
Griffith (1892). Physiology of the Invertebrata (New York).
MacMunn (1885). *Quart. J. Micro. Sci.* **28**, 469.
—— (1889). *Quart. J. Micro. Sci.* **30**, 51.
McLendon (1912). *J. Biol. Chem.* **11**, 435.
Needham, J. and Needham, D. (1926). *Proc. Roy. Soc. Lond.* B, **99**, 173.

XXV. THE INORGANIC PHOSPHATE AND A LABILE FORM OF ORGANIC PHOSPHATE IN THE GASTROCNEMIUS OF THE FROG.

By PHILIP EGGLETON and GRACE PALMER EGGLETON.

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(*Received December 31st, 1926.*)

In a study of the significance of phosphorus in muscle contraction (which will be reported separately) some observations were made which throw a doubt on the chemical results of some earlier workers. Evidence is given in this paper to show that the supposed inorganic phosphate of muscle is in certain conditions mainly organic phosphate of a very labile nature, which is so unstable in acid solution that it is hydrolysed during the estimation of the inorganic phosphate by the methods of Neumann, Embden [1921] or Briggs [1922]. Estimations performed in neutral or slightly alkaline solution, such as the Bell-Doisy method [1920], or precipitation by magnesia mixture, give results approximating to the truth, provided the muscle extract has not previously been exposed to the action of acid.

Briggs' method itself can be used to demonstrate the existence of this unstable phosphoric ester. Details of the method are given elsewhere: it suffices for the moment to say that after the addition of the appropriate reagents to a solution containing phosphate a blue colour develops, which rises to a maximum in about 30 minutes, after which time colour comparisons are usually made. It is easy to show that the rate of development of this blue colour follows a simple exponential law,

$$\text{intensity at time } t = c = P\,(1 - e^{-kt}),$$

where P is the final colour which measures the phosphate. The constant k has the value 0·12 (time measured in minutes). It follows mathematically (and can be proved experimentally) that in comparing two quantities of inorganic phosphate the ratio of the colour intensities will be the same at whatever time the comparison is made. If the colour ratio be plotted against time the result is a straight line parallel to the time axis (lines AB and CD in Fig. 1). A deproteinised extract from a dead muscle (killed by warming to 35° for 20 minutes) compared in this manner against an inorganic phosphate solution gives also a straight line, and is indistinguishable in this respect from an inorganic phosphate solution. When however a similar extract from a resting muscle is compared against an inorganic standard a different result

is obtained. The colour development begins by appearing equivalent to a very weak standard, but rapidly overtakes a much stronger standard. The result is a curve such as the curves *R* and *R'* in Fig. 1.

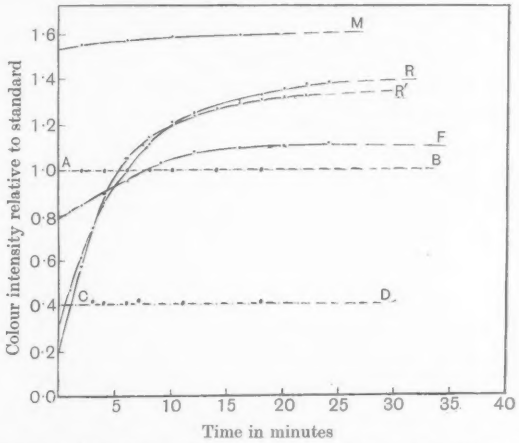

Fig. 1. The inorganic phosphate solution which gave the line *AB* contained 0·1 mg. of phosphorus. Another solution containing 0·04 mg. gave the parallel line *CD*. The remaining curves were given by muscle filtrates corresponding in each case to 160 mg. of muscle. The lines represent the colour intensity at different times relative to the standard solution which gave the line *AB*. (*R* and *R'*, resting; *F*, fatigued; *M*, in rigor.)

So complex are the conditions of colour development that little importance would attach to this fact were it not that a fatigued muscle shows this effect to a much less extent (curve *F*), whilst as has been said already, a muscle in rigor mortis shows no such effect at all (line *M*). Of the possible explanations which present themselves, the only one which survives the test of experiment is that there is an organic phosphate breaking down during the course of the estimation, causing the colour development curve to be pushed higher and higher. That this substance is completely destroyed in 20 minutes or so is shown by the curves in Fig. 2. In this type of experiment two portions of a resting muscle filtrate are treated simultaneously with Briggs' acid molybdate solution, but in one case the reducing solution is not added until 30 minutes later. In the latter case the colour production curve runs strictly parallel with an inorganic standard. The unstable organic phosphate has already broken down.

As to the nature of this unstable compound we have no evidence, save that it is concerned in muscular activity, and its disappearance coincides with fatigue. The name "phosphagen" suggests itself. "Phosphagen" might possibly be a phosphoric ester of glycogen, for traces of glycogen are present in trichloroacetic acid extracts of muscle. It is also possible that it may be the substance postulated by Meyerhof as a precursor for both lactic acid and "lactacidogen."

When Briggs' method is used to estimate the true inorganic phosphate, all that is necessary is to make colour comparison with the standard at frequent intervals, and obtain a curve such as those in Fig. 1. By extrapolating this curve backwards to zero time a value is obtained which represents the colour ratio which would have been given at the end of 30 minutes had the "phosphagen" been stable. The difference between this true value and that obtained after 30 minutes, measures the "phosphagen" in terms of the inorganic phosphate formed by its decomposition.

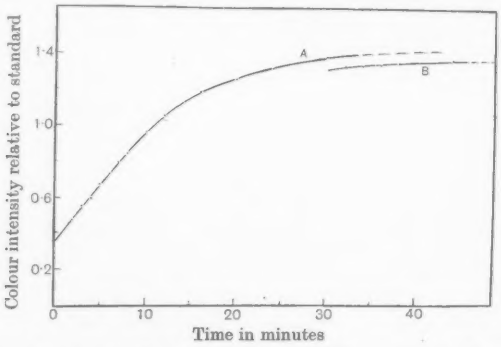

Fig. 2. The curve *A* represents the colour intensity at different times (relative to an inorganic standard started off at the same moment) given by an extract from a resting muscle, when the "inorganic" phosphate is estimated by Briggs' method. If the reducing agent is added 30 minutes after the acid molybdate reagent the colour production curve (*B*) is such as would be given by true inorganic phosphate. The "phosphagen" is already broken down. (The starting-point of the curve *A* is a measure of the true inorganic phosphate, whilst the height to which the curve rises is a measure of the "phosphagen.")

Table I. *Showing the rapid breakdown of "phosphagen" in acid solution.*

A fresh trichloroacetic acid filtrate from a resting muscle was estimated for inorganic phosphate and "phosphagen." Part of the filtrate was neutralised with $NaHCO_3$ and the remainder not treated. Both portions were incubated for an hour at 27° and the estimations were repeated on each. Results given in mg. of phosphorus per 100 g. of muscle.

	Inorganic *P*	"Phosphagen"	Increase in inorganic *P*	Decrease in "phosphagen"
Initial	27	57	—	—
After 1 hour at 27°	51·5	33	24·5	24
Incubated in presence of $NaHCO_3$	30·5	50	3·5	7

We have used 4 % trichloroacetic acid as a deproteinising agent, and have found that even in this solution the unknown substance disappears in a day or two. So rapid is this breakdown in the first hour or so, that special precautions must be taken to perform estimations as soon as possible after the removal of protein. Alternatively the extract can be neutralised with sodium bicarbonate, which renders the "phosphagen" less liable to breakdown on standing (Table I).

The stability of "phosphagen" in neutral solution led us to try the estimation of inorganic phosphate by the Bell-Doisy method, which is performed

in a mildly alkaline solution. The results (see Table II) agreed closely with the "corrected" values obtained by Briggs' method. In passing it should be mentioned that Bell and Doisy probably found traces of "phosphagen" in blood, for they state that "the inorganic phosphate must be determined in the trichloroacetic acid filtrate as soon as possible after filtering since the acid hydrolyses the organic phosphorus on standing, giving too high results. After standing 24 hours the values for inorganic phosphate and the total acid-soluble phosphorus are nearly identical." In the case of blood however the discrepancy appears to be a small one—not more than about 10 % of the value quoted for inorganic phosphate. In resting muscle extracts we have a discrepancy of the order of 300 %.

Estimation of the inorganic phosphate with magnesia mixture confirmed our belief that we were dealing with an organic phosphate unstable in acid. Although this method is notorious for giving high results (on account of the simultaneous precipitation of calcium and organic matter), our figures indicate that a resting muscle contains not more than 40 mg. % of inorganic phosphorus, less than half the figure given by the method of Embden (or any method involving the use of mineral acids).

Table II. *The "inorganic phosphate" of the frog's gastrocnemius, showing the falsely high values given by the Briggs and Embden methods, particularly for resting muscles.*

Exps. A, D, E and K were performed on batches of 6 to 8 frogs. In Exps. E, F and G the left gastrocnemius was used as a resting control on the right, which was stimulated through the nerve for 2 to 5 minutes with a supermaximal stimulation. The Briggs and Embden methods give high resting values which fall in fatigue. The other methods give low values which rise considerably in fatigue. All methods give the same high value for rigor.

		"Inorganic P" in mg. per 100 g. of muscle				
Reference		Embden	Briggs	Briggs corrected	Bell-Doisy	Magnesia mixture
Resting	A	92	87	—	—	—
	B	—	83	—	22	—
	C	—	91	18	—	—
	D	—	88	—	—	40
	E	95	86	—	—	—
	F	—	87	33	—	—
	G	—	84	28	27	—
	K	—	90	22	28	12
Fatigued	E	75	72	—	—	—
	F	—	73	56	—	—
	G	—	68	45	52	—
Rigor	H	—	105	102	—	—
	I	—	96	102	—	—
	K	—	104	90	108	103

Experimental.

The muscles were killed by immersion in liquid air and ground up with 4 % trichloroacetic acid in a mortar. The fluid was washed into an accurately calibrated cylinder and the volume made up with 4 % trichloroacetic acid to 4 or 5 cc. per 100 mg. of muscle. It was found essential to keep the temperature down to 0° until the actual estimation was performed; moreover the

fluid was filtered within 10 minutes of the maceration. Contrary to the results of certain other workers we have found it quite unnecessary to allow the extraction to continue overnight: extraction in 3 minutes was almost as complete as in 20 hours.

A quantity of filtrate calculated to contain about 0·1 mg. of phosphorus (inorganic plus "phosphagen") was diluted in a 15 cc. graduated flask to exactly 12 cc. An inorganic standard of equal or less strength contained in a similar flask was treated with the same amount of 4 % trichloroacetic acid and similarly diluted. The two reagents were added[1], and the solutions after being mixed were poured directly into the colorimeter cups. Readings could in this way be made within the first minute of the period of colour production. It is essential in following the colour production curves that the unknown and standard should be started off simultaneously and should be at the same temperature.

Where the Embden technique was used Embden's directions were followed in every detail, save that trichloroacetic acid was used to deproteinise the muscles. This is a simpler method and gives the same results as the Schenck method recommended by Embden.

The Bell-Doisy method was modified slightly to meet our special requirements. Instead of allowing 5 minutes for their acid-molybdate-quinol solutions to act before the addition of the sulphite-carbonate reagent, we reduced the time to about 1 minute for both muscle extract and inorganic standard.

The magnesia mixture used contained magnesium citrate instead of the chloride. This is supposed to give truer values.

Discussion.

We have found that the true inorganic phosphate content of a resting frog's gastrocnemius is of the order of 20 to 25 mg. of phosphorus per 100 g. of muscle. Estimations performed by the Briggs or Embden method give results of the order of 90 to 100 mg. per 100 g. in our hands (Embden's own figures are rather higher). This discrepancy of 70 mg. per 100 g. is attributable to a phosphoric ester which is very unstable in acid solution, and which is estimated as inorganic phosphate by the above methods. We have called this new compound "phosphagen."

In rapidly induced fatigue the true inorganic phosphate content is doubled at the expense of the "phosphagen," but considerably more "phosphagen" disappears than is necessary to account for the rise in inorganic phosphate. It is obvious that a method which estimates both inorganic phosphorus and "phosphagen" together should show a fall as a result of fatigue. We have found such a fall by both Embden's and Briggs' methods, though Embden

[1] Solution A: 5 % ammonium molybdate in 5·4 *N* sulphuric acid. Solution B: 0·5 % quinol in 20 % sodium sulphite. Solution B reduces the phosphomolybdate formed by the interaction of solution A with the inorganic phosphate.

himself claims to have found a rise [Embden *et al.*, 1922, 1925, 1926]. The figures quoted by Embden are so high, for both resting and fatigued muscles, that they cannot refer to the true inorganic phosphate.

When a muscle goes into rigor the "phosphagen" disappears entirely, and the inorganic phosphate rises to about four times the resting value. Here the "phosphagen" is more than accounted for by the inorganic phosphate which appears.

We desire to express our thanks to Prof. A. V. Hill and to Dr R. Robison for their suggestive criticism during the progress of the work, and to the Medical Research Council for a grant to meet the expenses.

REFERENCES.

Bell and Doisy (1920). *J. Biol. Chem.* **44**, 55.
Briggs (1922). *J. Biol. Chem.* **53**, 13.
Embden (1921). *Z. physiol. Chem.* **113**, 139.
Embden and Hentschel (1925). *Biochem. Z.* **156**, 343.
—— —— (1926). *Z. physiol. Chem.* **151**, 167.
Embden and Lawaczek (1922). *Biochem. Z.* **127**, 184.

XXVI. NOTE ON THE PREPARATION OF YEAST-JUICE BY BUCHNER'S METHOD.

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(*Received December 31st, 1926.*)

Some years ago (1920), before the yeast obtainable from breweries had regained its pre-war qualities, considerable difficulty was experienced in obtaining yeast-juice of a satisfactory degree of activity. It was, however, observed that, when the ground mass was kept for a few hours before being pressed out, the resulting juice was more active than that obtained by pressing at once. Accordingly a few experiments were made to test the reality of this effect and to ascertain under what conditions it was produced.

It was found that as a rule the juice obtained by pressing after an interval of 1–2 hours reacted much more vigorously with added phosphate than that pressed out at once.

This effect has been recently confirmed, although the yeast-juice obtained by direct pressing on this occasion was much more active than that prepared six years ago. Accordingly the preservation of the ground mass at air temperature for 2 hours before it is pressed out has been adopted as part of the routine of the preparation. Preservation of the expressed yeast-juice does not produce the same effect, so that it is probably due to some action involving constituents of the cell which are retained in the press cake. This question is being further investigated. All the experiments have been made with top-yeast from an English brewery.

Exp. 1. The ground mass was kept at air temperature and pressings made immediately after grinding and at intervals of 22 mins., 42 mins., 1 hr. 42 mins., 2 hrs. 42 mins., and 3 hrs. 42 mins. after grinding. Some of the juice from the first pressing was also kept at air temperature for 2 hours and then examined.

In each case two quantities of 25 cc. yeast-juice + 1 g. glucose + 0·2 cc. toluene were incubated at 25° for 35 mins. and the rate of fermentation observed. 2·5 cc. 0·6 *M* K_2HPO_4 + 2·5 cc. H_2O were then added to one of these quantities and 5 cc. 0·6 *M* K_2HPO_4 to the second and the rate of fermentation again observed.

The results were as follows:

4/11/20

Interval between grinding and pressing	Initial rate cc. per 5 mins.	After addition of 2·5 cc. 0·6 *M* K_2HPO_4		After addition of 5 cc. 0·6 *M* K_2HPO_4	
		Max. rate cc. per 5 mins.	Total in 45 mins.	Max. rate cc. per 5 mins.	Total in 45 mins.
0	1·6	3	23·4	2·4	11·5
22 mins.	3·3	4·6	29·8	3	24·5
42 mins.	3·1	4·6	34	8·7	35·6
1 hr. 42 mins.	3·6	6·1	44·8	5·6	36·2
2 hrs. 42 mins.	2·8	6·1	39·8	4·6	37
3 hrs. 42 mins.	3	5	40·9	3·6	29
Juice preserved for 2 hrs.	1·6	3·2	20·6	2·4	13·4

Exp. 2. The experiment was similar to Exp. 1 but only two pressings were made and three different quantities of phosphate (in this case a mixture of 8 vols. of 0·6 *M* KH_2PO_4 with 2 of 0·6 *M* K_2HPO_4) were used.

Interval between pressing and grinding	Initial rate cc. per 5 mins.	No addition Total in 45 mins.	After addition of phosphate: 5 cc. Max. rate	5 cc. Total in 45 mins.	10 cc. Max. rate	10 cc. Total in 45 mins.	15 cc. Max. rate	15 cc. Total in 45 mins.
0	1·65	8·2	9·5	78·3	5·4	46·4	4·8	28·6
2 hrs.	1·5	10·8	19·3	85·8	16·1	111·8	8·3	61

Summary.

In preparing yeast-juice by the grinding method from English top-yeast it is usually advantageous to preserve the ground mass for two hours at air temperature before pressing out the juice.

XXVII. THE AEROBIC AND ANAEROBIC METABOLISM OF THE COMMON COCKROACH (*PERIPLANETA ORIENTALIS*). II

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(*Received December 31st, 1926.*)

It has recently been shown [Davis and Slater, 1926] that when the common cockroach (*Periplaneta orientalis*) is placed in an atmosphere of nitrogen or some other inert gas, it goes into debt for oxygen, in a manner similar to that which has been observed in the case of striated muscle. This phenomenon was thought to be worthy of further investigation, in the hope that the superficial similarity might prove to be the expression of a common fundamental metabolism.

The metabolic process involved in the case of vertebrate muscle has been shown by Hill, Meyerhof and their co-workers to consist of two separate reactions, viz. (1) an initial breakdown of glycogen to lactic acid, and (2) the transformation of a portion of the lactic acid back again into glycogen, at the expense of the combustion of the remainder. The first reaction is anaerobic, and is the source of energy in the organism, the second is aerobic, and serves simultaneously as a means of removing the lactic acid, and of conserving the energy available in the glycogen. The efficiency of the total process depends largely upon the ratio of the lactic acid returned to glycogen to the lactic acid burnt. During oxygen lack the tissue obtains its energy in the normal manner, the lactic acid being allowed to accumulate. When oxygen again becomes available, this lactic acid is removed by synthesis and oxidation, the organism using an excess of oxygen, equal to that which it would have used during the period of anaerobiosis. In the case of vertebrate muscle, the ratio of lactic acid burnt to that resynthesised to glycogen is given by Furasawa and Hartree [1926] as 1 : 4·4, from which it can be calculated that a debt of 1 cc. of oxygen corresponds approximately to 6 mg. of lactic acid.

Working upon the assumption that a similar process is involved in the metabolism of the cockroach, it is possible, knowing the oxygen debt, to calculate (1) the lactic acid which should be found in the tissue, and (2) the volume of carbon dioxide which would be evolved if the lactic acid were completely neutralised by carbonate. This latter figure will be identical with the carbon dioxide which is retained when the lactic acid is subsequently removed.

An investigation is at present being carried out with a view to estimating the lactic acid occurring in conjunction with a given oxygen debt after a period of anaerobiosis. The present communication deals with a more detailed study of the respiratory exchanges following anaerobiosis in order to show, firstly, whether carbon dioxide is retained, and secondly, if this proves to be the case, whether the volume of the retained gas bears any relationship to the calculated amount of lactic acid present.

It may appear at first sight that it would be simpler and more direct to measure the carbon dioxide evolved during anaerobiosis, but practically it is easier to compare over a given recovery period the excess oxygen used and the carbon dioxide retained.

Method.

The measurements were made by means of the micro-respiration apparatus previously described [Slater, 1926]. This enabled the oxygen uptake and the carbon dioxide output to be determined simultaneously with an accuracy greater than 1 $mm.^3$.

Since the apparatus was originally described, some alteration has been made in the method adopted for ascertaining changes in the volume of the gases. The respiration vessel, in which the carbon dioxide is measured electrically by means of the Shakespear katharometer, is now connected to a small open manometer, instead of the original Haldane blood gas apparatus. At the bottom of the U of the manometer is a small rubber bag, which can be compressed by means of a plate and thumb screw. As the volume of the gases in the respiration chamber alters, the bag is compressed or relaxed, so as to bring the liquid on each side of the U back to the same level. By calibrating the arm of the U on the closed side, the changes in volume may be read at atmospheric pressure. To avoid errors which may be introduced during the experiment, by changes in barometric height or in the temperature of the thermostat, a second bulb as similar to the respiration vessel as possible and connected to a similar manometer, is placed in the thermostat alongside the actual apparatus. The changes in the volume of the air in this vessel are applied as a correction to the actual readings obtained for changes in the volume of the respired gas mixture. The new manometric arrangement has the advantage that it is small, and can be sealed directly on to the tube from the respiration vessel, thus avoiding all rubber joints, and permitting, if necessary, that the whole apparatus be shaken. This method of measuring volume changes is based upon that described by Warburg [1923].

The cockroaches were caught in the building, and were used immediately (except in those cases where they were kept for a few days on a glucose diet in order to raise their respiratory quotient) to ensure that they were in no way damaged by being kept in captivity. The respiratory quotient of the freshly caught insects was rarely above 0·85, probably due to the fact that

in the building where they were caught, there was abundant food of fat and protein, but hardly any carbohydrate.

The insect selected for the experiment was weighed, and dropped into the lower part of the respiration vessel. Care was taken in attaching this to the upper portion that the antennae were not trapped in the ground glass joint. The apparatus was then placed in a thermostat kept at 25°, and left until the cockroach had settled into a quiet state. When the necessary conditions had been attained an oxygen-carbon dioxide mixture was led through the apparatus until the reading of the katharometer showed that all the air had been expelled. The taps were then closed and readings taken of the carbon dioxide percentage and the total volume. When the readings became steady they were continued for an hour in order to get a value for the resting metabolism under the conditions of the experiment. A stream of nitrogen was next passed through the apparatus until the cockroach ceased to struggle. The vessel was then closed for from 30 mins. to 1 hour. As will be seen later, for purposes of comparison it is not necessary that the anaerobiosis should be absolute. Such a condition would be difficult to obtain with the type of apparatus which was in use. At the end of the anaerobic period the nitrogen was rapidly replaced by the oxygen-carbon dioxide mixture, and readings commenced as soon as possible and continued until the respiratory quotient again reached its normal value.

Results.

In Table I are given the chief details of 7 experiments.

Table I.

Experiment	1	2	3	4	5	6	7
Weight of cockroach (g.)	0·13	0·07	0·19	0·22	0·18	0·12	0·08
Condition of cockroach	Glucose-fed	Freshly caught	Freshly caught	Freshly caught	Freshly caught	Glucose-fed	Starving for 1 month
% carbon dioxide at the beginning of the experiment	1·70	1·76	1·44	1·47	0·83	0·60	0·35
Normal respiration:							
Oxygen mm.³ per g. per hour	314	375	292	258	398	366	225
Carbon dioxide mm.³ per g. per hour	315	365	242	241	277	342	200
Post anaerobic respiration (period of excess oxygen intake):							
Oxygen mm.³ per g. per hour	370	433	350	359	458	416	288
Carbon dioxide mm.³ per g. per hour	262	384	249	260	395	346	209
Excess oxygen used mm.³ per g. per hour	56	58	58	101	60	50	63
Carbon dioxide retained mm.³ per g. per hour	108	39	51	82	32	46	54

In the table the rise in oxygen intake after anaerobiosis is clearly marked in each experiment, but this value is not in proportion to the time during which the cockroach was in nitrogen. This is due to the difficulty experienced

in obtaining the complete removal of oxygen by the method used. The nitrogen always contained traces of oxygen, and the shape of the respiration vessel rendered the complete removal of the gases previously present a matter of considerable difficulty. As however the results are used only for the comparison of the oxygen debt replacement and the carbon dioxide retention over the same period of recovery, the efficiency of anaerobiosis is immaterial, provided always that it does not vary outside those limits which may be expected to produce profound tissue derangement.

It was shown [Davis and Slater, 1926] that the normal oxygen uptake at 25° was about 500 mm.^{3} per g. per hour. In the present experiments the values obtained are all below this average figure. No positive explanation of this difference can be advanced, but it is tentatively suggested that it is due to the fact that the present experiments were carried out at a definite carbon dioxide pressure. Enough data are not available in support of this view to render it more than a working hypothesis, although the results of the experiments in Table I agree with it as far as they have been carried. With the exception of Exp. 7, where the cockroach was distinctly abnormal, owing to being without food for over a month, the oxygen intake appears to be reduced with higher percentages of carbon dioxide. (It is to be noted that in Exp. 7, the weight of the insect is only 0·08 g., *i.e.* it is not fully matured and hence according to previous determinations its oxygen uptake should be approximately 600 to 700 mm.^{3} per g. per hour.)

To obtain the carbon dioxide retention during the post anaerobic phase, it is necessary to calculate the carbon dioxide which should have been evolved during this period. This value is easily obtained if we assume that the normal metabolism is going on with the recovery process superadded; for under these conditions the carbon dioxide evolved will be equal to the normal plus that from the oxidation of the lactic acid. The combustion of the lactic acid has a unit respiratory quotient, and hence the carbon dioxide evolved is equal to the excess oxygen taken in. Thus we have

$$CO_2 \text{ output after anaerobiosis} = \text{Normal } CO_2 \text{ output} + O_2 \text{ debt.}$$

The carbon dioxide retained, as given in the last row of figures in Table I is obtained by subtracting the output found from the output calculated in this way. The retention of carbon dioxide is clearly shown in each experiment. In Table II are shown the respiratory quotients for the same experiments, again illustrating the carbon dioxide retention.

Table II.

Experiment	1	2	3	4	5	6	7
Normal R.Q.	1·00	0·98	0·83	0·93	0·70	0·93	0·89
Post anaerobic R.Q.:							
Calculated	1·00	0·98	0·86	0·95	0·73	0·94	0·91
Found 1st half hour	0·60	0·87	0·72	0·65	—	—	—
2nd ,,	0·83	0·91	0·68	0·63	0·62	0·82	0·53
3rd ,,	1·03	0·98	0·73	0·74	0·71	0·83	0·95
4th ,,	0·96	0·97	0·83	0·83	0·81	—	0·78

It will be seen that in the majority of cases, the respiratory quotient has returned to normal at the end of 2 hours. The figures given for the calculated respiratory quotient for the post anaerobic period allow for the excess oxygen used liberating its own volume of carbon dioxide.

There remains now only the consideration of the relation between the oxygen debt and the carbon dioxide retained. If the figure given above—1 cc. of oxygen equivalent to 6 mg. of lactic acid—is used to calculate the lactic acid present in the tissue, the results shown in row 3 of Table III are obtained.

Table III.

Experiment	1	2	3	4	5	6	7
Excess oxygen mm.^{3}	56	58	58	101	60	50	63
Lactic acid corresponding to excess oxygen, mg.	0·336	0·348	0·348	0·606	0·360	0·300	0·378
Carbon dioxide retention calc. from lactic acid, mm.^{3}	84	87	87	151	90	75	94·5
Carbon dioxide retention found, mm.^{3}	108	39	51	82	32	46	54
Ratio CO_2 found/CO_2 calc.	1·2	0·46	0·59	0·54	0·36	0·60	0·57

From the lactic acid, the volume of carbon dioxide which should be retained during the recovery process can be calculated, and is given in row 4. The accuracy of these figures depends on two unknown factors, viz. (1) the extent to which carbonates are involved in the neutralisation of the lactic acid, and (2) the efficiency of the oxidative recovery process. For purposes of the calculation it has been assumed that the whole of the neutralisation is by carbonates, and that the efficiency of the recovery process is the same as that of striated muscle. It is improbable that the first assumption is correct, and there is no precedent for the second. Fortunately these unknown factors will affect all the results proportionately, and if we determine the ratio between the carbon dioxide found and that calculated, it should, on the assumption that the carbon dioxide retention is due to the removal of the lactic acid, be approximately constant. This ratio is given in row 5, and, with the exception of Exp. 1, the values lie between 0·36 and 0·60, with a mean of 0·52, and a percentage mean deviation of 14·6. For the type of experiment under consideration, the agreement is sufficiently good to lend support to the view that the oxygen debt and the carbon dioxide retention are related through the removal of accumulated lactic acid. In the case of Exp. 1 it can only be suggested that some unknown error has been introduced, but as it was the only experiment which fell outside the series, it was considered reasonable to neglect it for the time being.

Summary.

(1) After a period of anaerobiosis the cockroach not only uses an excess of oxygen, but also retains a considerable volume of carbon dioxide.

(2) The oxygen debt and the carbon dioxide retention are shown to be related through the removal of lactic acid.

The expenses of this research were largely defrayed by a grant from the Royal Society.

REFERENCES.

Davis and Slater (1926). *Biochem. J.* **20**, 1167.
Furasawa and Hartree (1926). *J. Physiol.* **62**, 203.
Slater (1926). *J. Sci. Instruments*, **3**, 177.
Warburg (1923). *Biochem. Z.* **142**, 317.

XXVIII. SOME PHOSPHORUS COMPOUNDS OF MILK. III.

DEPHOSPHORISED CASEINOGEN. THE ACTION OF ALKALI UPON CASEINOGEN.

By CLAUDE RIMINGTON

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From the Biochemical Laboratory, Cambridge.

(*Received December 31st, 1926.*)

The name "dephosphorised caseinogen" was given by Rimington and Kay [1926] to the protein produced from caseinogen by the action of 0·25 *N* sodium hydroxide for 24 hours at 37°. This substance contains only minimal amounts of phosphorus but in other respects does not differ greatly from caseinogen. Preliminary experiments indicated, however, that dephosphorised caseinogen possesses a low nitrogen content, values lying between 11·1 % and 13·1 % being found for samples prepared from different brands of commercial "casein," the total nitrogen of caseinogen itself being 15·65 %.

During the reaction with sodium hydroxide a considerable evolution of ammonia occurs and it was with the object of ascertaining the source of this ammonia that the following investigations were undertaken.

Dephosphorised caseinogen, obtained from a specimen of caseinogen prepared from separated milk, was analysed by the nitrogen distribution method of Van Slyke, employing the modifications recently suggested by Plimmer and Rosedale [1925]. The analyses were performed in duplicate. In Table I are

Table I. *Distribution of nitrogen in dephosphorised caseinogen.*

Total nitrogen = 11·06 %, total phosphorus = 0·009 %.

		Caseinogen				Dephosphorised caseinogen		
	Error of the method (Van Slyke) % of total nitrogen	Crowther and Raistrick % of total nitrogen	Plimmer and Rosedale % of total nitrogen	Van Slyke % of total nitrogen	Caseinogen Mean of last three columns	Mean of two analyses % of total nitrogen	Dephosphorised caseinogen as percentage of total N – amide N	Caseinogen as percentage of total N – amide N
Amide-N	0·37	10·25	10·63	10·35	10·41	4·35	—	—
Humin-N	0·39	1·26	1·08	2·35	1·56	1·80	1·88	1·74
Total N of bases	—	26·62	22·68	22·85	24·05	27·25	28·49	26·84
Arginine-N	1·27	9·31	7·74	7·46	8·17	6·82	7·13	9·12
Histidine-N	0·93	6·55	5·54	5·23	5·77	7·03	7·35	6·44
Lysine- + cystine-N	1·23	10·76	9·41	10·16	10·11	13·39	14·01	11·28
Total N of filtrate	1·50	62·31	62·58	63·75	62·88	64·13	67·05	70·16
Amino-N of filtrate	1·10	55·44	51·93	55·43	54·27	60·21	62·95	60·56
Non-amino-N of filtrate	1·20	6·87	10·66	8·32	8·62	3·92	4·10	9·60

recorded the mean results expressed as percentages of total nitrogen, together with some previous analyses of caseinogen to be found in the literature. The figures given by Plimmer and Rosedale have been recalculated for expression as percentages of total nitrogen. In order further to facilitate the comparison of dephosphorised caseinogen with caseinogen, the nitrogen distribution in these two proteins has also been calculated in such a way that the nitrogen of each fraction is expressed as a percentage of the total nitrogen of the protein less the amide nitrogen.

From these figures it will be seen that the amide nitrogen of dephosphorised caseinogen is distinctly lower than that of caseinogen. There is also some decrease in the arginine nitrogen accompanied by an increase in the lysine + cystine fraction of the former. Arginine, in the free state, is known to be attacked by hot sodium hydroxide with the liberation of ornithine and ammonia and it is highly probable that a similar change has here taken place. The resulting ammonia would be evolved along with that derived from the amide nitrogen groups whilst the other product, ornithine, would become included in the figure for lysine + cystine nitrogen.

The nature of the groups giving rise to what is termed amide nitrogen on acid hydrolysis is not known with certainty, although there is evidence in support of the view put forward by Osborne that this nitrogen is present in the protein in the form of amides of the dicarboxylic acids; and in the particular case of caseinogen, Luck [1924] has succeeded in isolating a dipeptide of lysine and glutamine in which this linkage is present. It must be admitted however that the substance isolated by Luck represents only a portion of the groups capable of yielding ammonia when caseinogen is subjected to hydrolysis.

The ease with which a portion of the amide nitrogen fraction of caseinogen is converted into ammonia by the action of relatively weak alkali at 37° is a point of some interest and is in keeping with the supposition that acid amide groups exist in the protein.

It will be seen however that there still remains some 4 % of the total nitrogen of dephosphorised caseinogen in the form of amide nitrogen and in order to ascertain whether this also could be removed by the prolonged action of sodium hydroxide, of the same strength as previously employed, the following experiments were performed in which the rate of ammonia liberation was followed.

Equal volumes of a 4 % solution of caseinogen and of 0·5 *N* sodium hydroxide, both previously warmed to 37°, were mixed and a sample at once taken for total nitrogen determination, the remainder being transferred to a well-stoppered bottle, held in a thermostat at 37° and containing a little paraffin to prevent foaming. A rapid stream of air was then drawn through the solution and the liberated ammonia collected in standard acid, the excess of acid being titrated at suitable intervals and the ammonia determined by difference.

The result of such an experiment is shown in Fig. 1.

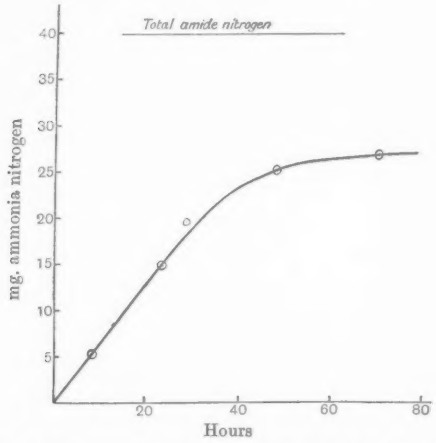

Fig. 1. Liberation of ammonia from caseinogen by 0·25 *N* sodium hydroxide at 37°.

There is a rapid liberation of ammonia during the first 30 hours, at the end of which time approximately 50 % of the total amide nitrogen has been eliminated. During this initial stage the velocity of ammonia production remains constant but subsequently falls. At the end of 70 hours the reaction has almost ceased, there being still however a very gradual liberation of ammonia not amounting to more than 0·5 mg. in 24 hours.

The total ammonia production up to this point is equivalent to about 65 % of the total amide nitrogen. The remainder appears to be comparatively stable towards alkali of the strength employed in these experiments.

Dephosphorised caseinogen gives all the colour tests which are given by caseinogen. It is insoluble in water but soluble in sodium hydroxide, requiring approximately the same quantity of alkali as caseinogen to effect complete solution. It is only very slowly attacked by proteolytic enzymes, a circumstance which may be due to some racemisation having taken place during its preparation by action of the alkali upon the protein. It is not coagulated by rennin.

Summary.

1. The low nitrogen content of dephosphorised caseinogen in comparison with caseinogen is accounted for by its much lower content in amide nitrogen.

2. During the action of sodium hydroxide upon caseinogen, ammonia is evolved, derived chiefly from the groups giving rise to amide nitrogen on acid hydrolysis, but also to some extent as a result of the decomposition of arginine.

3. Dephosphorised caseinogen is only slowly attacked by proteolytic enzymes. It is not coagulated by rennin.

The writer desires to record his gratitude to Sir F. G. Hopkins for his continual interest and advice. His thanks are also due to the Department of Scientific and Industrial Research for a grant held throughout the course of this work.

REFERENCES.

Luck (1924). *Biochem. J.* **18**, 679.
Plimmer and Rosedale (1925). *Biochem. J.* **19**, 1004.
Rimington and Kay (1926). *Biochem. J.* **20**, 777.

XXIX. THE EFFECT OF EXCESSIVE RADIATION WITH ULTRA-VIOLET LIGHT UPON THE GROWTH OF RATS.

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(*Received January 1st, 1927.*)

A series of experiments was made in this Institute [Chick and Roscoe, 1926] in which the vitamin A content of milk was titrated by a method based on that of Steenbock, Nelson and Black [1924]. In this method the milk doses were given after growth had ceased upon the defective diet, but the vitamin D requirement of the rat was satisfied by means of daily irradiation with ultra-violet light for 10 minutes or longer. This irradiation was continued during the test period.

It was found that the growth which had been restored by addition of milk to the diet, tended to diminish after 3 to 4 weeks, and more quickly if the time of irradiation was increased from 10 to 20 minutes daily. At the same time the rats developed conjunctivitis with purulent secretion.

An excessive dose of irradiation was clearly deleterious, and it was suggested to me by Dr Chick that it might be worth while to determine whether the effect was entirely due to the conjunctivitis, and also whether it was enhanced when the animals' diet was defective.

The following two experiments were therefore designed to determine:

(1) whether there is a maximum period of time and intensity up to which irradiation with ultra-violet rays is beneficial to growth, and beyond which it is deleterious;

(2) whether the presence or absence of vitamin A in the diet has any influence on the supposed deleterious effects of prolonged exposure to such irradiation.

Technique.

Young rats of 40–50 g. weight received a diet, "F" diet [Chick and Roscoe, 1926], complete in all other respects, but devoid of fat-soluble vitamins. In exp. 1 this was supplemented in some cases by daily doses of pasture-fed butter, to provide vitamin A. In exp. 2 the rats received the same diet without supplement of butter.

The mercury vapour quartz lamp was made by the Hewittic Electric Co., with a straight burner 10 cm. in length; on a 200 volt circuit it was using a current of 4 amperes. It had been in use for about 2 years at the beginning

of the experiment. The rats were exposed at a distance of 42 cm. from the lamp.

Exp. 1. The rats were divided into groups receiving irradiation from the lamp for 2′, 5′, 10′ and 30′ respectively. Each group contained 6 rats of which 3 received 0·2 g. of butter daily, and 3, 0·5 g. In addition there was a group of 2 rats receiving 1·0 g. of butter and 30′ irradiation and control rats which received 0·5 g. of butter and were not irradiated.

The first experiment was begun without arranging for protection to the rats' eyes. After about 12 days, traces of purulent secretion were observed in the eyes of some rats receiving 10 to 30 minutes' irradiation, and steps were at once taken to shield them. This was effected by means of a small circular wash-leather mask with a hole in the centre. The snout was passed through the hole. The mask covered the rats' eyes. A bag of wide-meshed mosquito-net was stitched to the mask and tied firmly behind the head and also behind the fore-legs with strings of narrow tape threaded through the meshes of the mosquito-net. The open end of the bag was secured firmly by safety-pins behind the hind-quarters and this prevented the rats from wriggling out.

With this protection the condition and the eyes improved rapidly and in the course of a week or 10 days the appearance was again normal. Later on, after 4 to 6 weeks, it was noticed that the eyes became sore and showed a blood-stained purulent secretion. The time of onset did not appear to depend upon the length of daily exposure to the ultra-violet radiation; but it was noticed that the rats affected were those whose eyes had been unprotected at the beginning of the experiment, the others continuing to show healthy eyes. This affection of the eyes may have been due to a slightly incomplete supply of vitamin A, but the deficiency must have been very slight, especially in the case of rats receiving the larger doses of butter. The exposure of the eyes at a previous period seemed to have rendered them hyper-sensitive to some slight defect.

It is conceivable that this abnormality may have been concerned with an insufficiency of vitamin B. McCollum, Simmonds and Becker [1925] describe an ophthalmia indistinguishable from that caused by vitamin A deficiency, but which they attribute to an insufficiency of vitamin B, combined with a badly balanced salt mixture in the diet. Hume (verbal communication) has also observed a slight degree of ophthalmia in rats on diets well supplied with vitamin A, which she attributes to an insufficiency of vitamin B.

There was no significant difference to be detected in the first experiment as regards growth in weight between the animals in the different groups, with the exception of the male control which was considerably below the average weight of the irradiated male rats. Disregarding a few exceptions evenly distributed in the various groups, growth was sustained throughout the observation, which lasted 12 to 13 weeks, and there was no failure in the case of rats receiving the largest doses of irradiation. The smallest dose of butter (0·2 g.) appeared to be as effective as the larger doses.

Exp. 2. The rats were divided into three groups, one group of 2 rats receiving no irradiation and two groups of 3 rats receiving daily irradiation for 2′ and 30′ respectively. These rats received no supplement to the "F" diet. The eyes were protected during irradiation of the animal from the beginning of the observation.

As was to be expected, the irradiated rats grew better than the controls but there was no significant difference in this respect between the two groups of irradiated animals. Seven out of the eight rats displayed a more or less marked degree of xerophthalmia, the onset of which was earlier in the majority of the irradiated rats than it was either in their own controls or in the irradiated rats receiving butter (exp. 1). The difference was not however very striking and as the one rat in which the eyes remained normal was an animal receiving the maximum dose of irradiation, the experiment does not seem to suggest that these symptoms of vitamin A deficiency were aggravated by exposure to ultra-violet light.

Summary and Conclusions.

(1) Previous observations in which ultra-violet radiation had exercised a deleterious effect upon the health of experimental rats are confirmed but the effect is found to be due to the conjunctivitis from which the animals suffered.

(2) Provided that the eyes were shielded, exposure of young growing rats to ultra-violet radiation for periods up to 30′ daily was found to have no deleterious effect upon the growth and well-being of the rats (although this dose was found to be 15 times the adequate dose for male rats). It was also found that exposure for 30′ to the lamp had no more beneficial results than exposure for the shorter periods.

(3) When using the biological method for the titration of vitamin A it is preferable to provide vitamin D by the inclusion of an irradiated vitamin-free rat in the diet, than by direct irradiation of the test rats.

Thanks are due to Dr H. Chick, Prof. C. J. Martin and Miss E. M. Hume, for kind help and criticism, and to the Lister Institute for permission to carry out the experiments there.

References.

Chick and Roscoe (1926). *Biochem. J.* **20**, 633.
McCollum, Simmonds and Becker (1925). *J. Biol. Chem.* **64**, 161.
Steenbock, Nelson and Black (1924). *J. Biol. Chem.* **59**, *Proc.* ix.

XXX. A FURTHER NOTE ON THE ANTI-RACHITIC VALUE OF FRESH SPINACH.

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(*Received January 3rd, 1927.*)

The presence of the antirachitic vitamin D in green leaves has been a matter of considerable uncertainty. McClendon and Shuck [1923], and Zucker and Barnett [1923], in dietetic experiments with rats receiving low phosphorus rachitic diets, could not detect any antirachitic properties in spinach leaves.

On the other hand, cut green leaves irradiated by the mercury vapour lamp have been shown to acquire active antirachitic properties [Hess and Weinstock, 1924, 1925; Chick and Roscoe, 1926, 1], and it seemed probable that the same synthesis would take place to some extent in leaves exposed to the ultra-violet rays in the sunlight. This has however never been conclusively proved.

Chick and Roscoe [1926, 1] showed that additions of fresh, summer-grown spinach to a diet adequate in calcium and phosphorus produced a slight improvement in calcification of the bones of young rats. No improvement was obtained with spinach grown in the winter. The observations of Luce [1924], and Chick and Roscoe [1926, 2], working with a milch-cow, gave some indication that the antirachitic value of the milk was slightly higher when the cow, itself exposed to summer sunshine, was being fed on green fodder than when it was fed on dry food.

Chick and Roscoe [1926, 1] have put forward two theories to account for the discrepancy between the antirachitic value of spinach exposed to the ordinary sunshine and that irradiated by ultra-violet light from an artificial source. (i) That the antirachitic vitamin is present in such small amounts that the effect could not be demonstrated with the quantities hitherto employed. (ii) That though the vitamin is synthesised in the green leaves it is rapidly removed or destroyed.

To examine he latter hypothesis it was decided to supplement the vitamin D-deficient diet of a set of rats with spinach picked in the open at mid-day and immediately fed to them.

To obviate the difficulty described under (i), rabbits were used in one experiment, since they will eat a much larger amount of green stuff in relation to the basal diet than will rats.

Experimental.

McCollum's No. 3143 low phosphorus, rickets-producing diet was used [McCollum, Simmonds, Shipley and Park, 1921]. With young rats on this diet a greater difference in bone calcification was found between those animals receiving the basal diet alone and those receiving the basal diet, supplemented with abundant vitamin D, given as cod-liver oil or by irradiation, than when a diet adequate in phosphorus was used. A better histological picture of rickets was also found.

The rabbits had the same diet, the only difference being that it was given as a dry mixture of ground cereals, gelatin and salts, etc. Water was given separately. It was eaten well, except that some of the gelatin was always left. Both control rabbits fed on this diet showed definite rickets.

The spinach given was of the prickly seeded variety (*Spinacea oleracea*). That given to the rats was grown in boxes on the roof of this Institute, during the months of June and July, 1926. It was picked and fed to the animals at mid-day, having been in what sunlight there was all the morning. There were not many days during the period without some sunshine. As this source was not adequate to supply the rabbits, they received bought spinach in addition. In some cases this was exposed to the sunlight, with the cut ends in water, for 3 to 4 hours before feeding ("sunned spinach"); in others it was fed as bought from the shop.

The largest average amount of spinach eaten per rat per day was 1·5 g. These rats were eating 15 g. of food daily. The dose therefore formed 10 % of the wet weight of the diet. The rabbits ate 40 g. of the spinach and about 40 g. of dry basal diet (equivalent to 160 g. of wet diet) daily. The spinach therefore formed 25 % of the wet weight of their diet. The rabbits would have eaten more spinach, but it was thought inadvisable to alter the ratio of calcium to phosphorus in the food to any greater extent; by the 25 % dose of spinach it was lowered from 4·1 : 1·0 in the basal diet, to 3·8 : 1·0 in the diet with spinach addition. This alteration cannot have been large enough to account for the results obtained [McCollum *et al.*, 1921].

Exp. 1. *Young rats.* Five male rats from one litter, approximately 40 g. in weight, were fed on McCollum's 3143 diet. One received the basal diet alone, three were given in addition as much spinach as they would eat, and the fifth was given cod-liver oil. At the end of four weeks they were killed, their leg bones analysed by the method described by Chick, Korenchevsky and Roscoe [1926], and a histological examination of their rib junctions made. The results are set out below.

		Ca % on fat-extracted dry weight of leg bones*		% increase in calcification		A/R ratio	
Control		8·61		—		0·303	
Spinach average per diem	0·8 g.	9·86	average = 9·49	11	average = 11	0·363	average = 0·348
	1·0 g.	9·06		10		0·348	
	1·5 g.	9·56		11		0·332	
Cod-liver oil 0·05 g. per diem		15·27		77		0·703	

* Calculated as 37 % of the ash.

All the rats, except the one receiving cod-liver oil, exhibited angulation of the rib junctions and fractures, but this was more severe in the negative controls than in the others.

The A/R ratio, or the ratio of the ash of the bones to the organic residue of the fat-extracted bone, is included as giving a very satisfactory criterion of bone calcification in young rats [Chick, Korenchevsky and Roscoe, 1926].

The proportion of calcium in the leg bones, reckoned on the fat-extracted weight, is increased from 8·6 % to 9·5 % or by about 11 %. This increased calcification is however far short of that achieved in the "positive control animal" which received 50 mg. of cod-liver oil per day and showed 15·3 % calcium in the fat-extracted bones. The A/R ratio is increased from 0·303 to 0·348.

A definite slight increase in calcification is noticeable. The histology shows severe rickets in all rats except the one receiving cod-liver oil, which was normal.

Exp. 2. *Young rabbits.* Six young rabbits of one litter were placed on the basal diet at the age of 4 weeks, when weighing approximately 300 g. each. Four of these received a supplement of fresh spinach leaves, in two cases as it was bought from the shop, in two after it had been exposed to sunshine. At the end of 5 weeks they were killed, the femurs of both legs analysed and the rib junctions examined histologically. The results are set out below.

	Ca % on fat extracted dry wt. of femur*		% increase in calcification		A/R ratio		Histology	
	♂	♀	♂	♀	♂	♀	♂	♀
Controls	15·78	19·15	—	—	0·745	1·029	Definite rickets	Slight rickets
Spinach 40 g. per diem (as bought)	17·12	18·64	11	−3	0·845	1·068	Slight rickets	Very slight rickets
Spinach 40 g. per diem (sunned)	16·84	20·73	11	11	0·826	1·226	Slight rickets	Normal

The control animals exhibited macroscopic rickets with swollen rib junctions; the ribs of the male control had also several fractures.

If the rabbits receiving spinach be compared only with the controls of their own sex, it will be seen that in three out of the four cases the percentage calcification of their bones is raised, that the calcification as measured by the A/R ratio is raised in all cases, and that the histology is also improved in all cases. The animals receiving sunned spinach do not show a consistent improvement over those receiving the unsunned, either in calcification or in histology.

The calcification in the males is consistently lower than in the females and the histological rickets more severe. This, if always so, constitutes a serious difficulty in the setting out of experiments with young rabbits as it is practically impossible to determine the sex at four weeks.

* The Ca was determined directly by a modification of McCrudden's method [Boas, 1924]. It was found to be equal to 37 % of the ash in rabbits as in rats.

Discussion.

All but one of the animals receiving spinach showed a slight increase in the calcium content of the bones compared with the control animals. Taking the A/R ratio as the criterion of calcification the increase is consistent. This confirms the previous results of Chick and Roscoe [1926, 1] with summer-grown spinach.

No greater percentage increase in the calcium content of the bones was observable in the rabbits, 25 % of whose diet was spinach, than in the rats, 10 % of whose diet was spinach. This may be due to differing requirements of rats and rabbits for vitamin D. At the same time it may be noted that the calcium content of the bones of the rat which received 1·5 g. of spinach was no greater than that of the one which received 0·8 g.

Histologically, whereas the rats all showed rickets to such a marked degree than no distinction could be made between the controls and those receiving spinach, the rabbits receiving spinach showed an improvement from pronounced to slight rickets in the males, and from slight rickets to normal in the females.

The presence of vitamin D in small amounts in fresh spinach is therefore demonstrated. Improvement in the histology is shown better in experiments with rabbits than with rats. This is probably due to the larger amount of spinach eaten.

These experiments do not afford any evidence in favour of the theory that vitamin D, after synthesis in the leaf, is rapidly removed. In Exp. 2, carried out with spinach leaves which, with the cut ends in water, were placed in the sun immediately before being given to the animals, the results were no better than when the spinach supplement was not so treated. In Exp. 1, carried out with leaves which were on the plant in the sun till the time of feeding, calcifying power was demonstrated, but this was no greater than that obtained by Chick and Roscoe [1926, 1] using the same quantities of leaves picked in the evening of the day before they were fed.

Summary.

1. A slight but definite influence upon calcification is again demonstrated by adding fresh green leaves of summer spinach to the diet of young rats and rabbits.

2. This effect is more obvious in experiments with rabbits than with rats.

3. The difference of opinion as to the presence of vitamin D in green leaves is probably due to the difficulty of demonstrating the small amounts contained.

My thanks are due to Prof. C. J. Martin and to Dr H. Chick for their advice and criticism during this research, and to Prof. Korenchevsky for help with the histology.

REFERENCES.

Boas (1924). *Biochem. J.* **18**, 425.
Chick, Korenchevsky and Roscoe (1926). *Biochem. J.* **20**, 622.
Chick and Roscoe (1926, 1). *Biochem. J.* **20**, 137.
—— —— (1926, 2). *Biochem. J.* **20**, 632.
Hess and Weinstock (1924). *J. Biol. Chem.* **62**, 301.
—— —— (1925). *J. Biol. Chem.* **63**, 297.
Luce (1924). *Biochem. J.* **18**, 716, 1297.
McClendon and Shuck (1923). *Proc. Soc. Exp. Biol. Med.* **20**, 288.
McCollum, Simmonds, Shipley and Park (1921). *J. Biol. Chem.* **47**, 507.
Zucker and Barnett (1923). *Proc. Soc. Exp. Biol. Med.* **29**, 375.

XXXI. OPTICAL ROTATORY POWER AND DISPERSION OF PROTEINS.

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(*Received January 1st, 1927.*)

The importance of optical rotatory power for the characterisation of proteins has received attention since 1880 [Fredericq, 1880; Starke, 1881; Sebilin, 1885].

There is however a wide divergence in the results obtained; *e.g.* in the case of serum albumin values for the specific rotatory power for the D-line vary from − 33° [Reiss, 1904] to − 63° [Young, 1922]; other values being − 47·8° [Maximovitsch, 1901], − 42° to − 49° [Gürber, 1894], − 48° [Michel, 1895], − 51° to − 66° [Lewis, 1922]. Similarly in the case of serum globulins, values varying from − 42° [Porges and Spiro, 1902] to − 51·2° [Seng, 1899] and − 52° [Lewis, 1922] have been recorded. This divergence alone renders it desirable that the data be critically examined and certain of the determinations repeated. Also, until a recent publication of Hafner [1925] which appeared after the present work had commenced, no attention has been paid to the rotatory power of the proteins for light of different wave-lengths, *i.e.* their optical rotatory dispersion.

From many points of view we consider this work of Hafner to be unsatisfactory. Like all other workers in this field except Hardy and Gardiner [1910] and Young [1922] lipins were not removed from the proteins although these considerably modify their properties. In addition Hafner made no attempt to separate euglobulin and pseudoglobulin although they have different rotations; and the use of very dilute solutions for optical measurements and the lack of duplicate determinations make the results even less dependable. As will be shown later, the results are not acceptable on other experimental and theoretical grounds.

The optical rotation and dispersion of lipin-free serum and other proteins have therefore been determined.

Experimental methods.

Serum proteins.

Lipins were extracted from serum by a modification of Hardy and Gardiner's method [1910] and that of Hartley [1925]. Plasma, or preferably serum (100 cc.), at 0° is poured into a mixture (600 cc.) of alcohol (7 parts) and ether (3 parts) cooled to − 15°. The mixture is vigorously stirred and the

temperature not allowed to rise above $-5°$. After allowing to stand at this temperature in an ice-chest for 2 hours the precipitated proteins are filtered off on a Büchner funnel in the ice-chest, washed several times with portions (40 cc.) of the alcohol-ether mixture at 0°, and with several portions (50 cc.) of ether at 0°. The filtration accelerates markedly when the ether washing is commenced. The proteins are then rapidly transferred to extraction thimbles and extracted for 2 hours in a Soxhlet apparatus with ether, metallic sodium being placed in the extraction flask to remove alcohol extracted from the proteins. After 2 hours' extraction the ether and sodium in the apparatus are renewed and the extraction is continued for 20 hours. It is found that if the proteins are dried *in vacuo* before ether extraction, an appreciable amount of alcohol remains in the precipitate and considerable denaturation occurs. After completion of the ether extraction the proteins are transferred to a desiccator and dried *in vacuo* for 48 hours. The dried proteins are not denatured by standing in contact with a trace of ether as they are by alcohol. When freed from ether the proteins are allowed to stand for a while in contact with laboratory air to become slightly moist, otherwise, when completely dry, they are difficult to work with, easily electrified and adhere to surfaces. Obtained in this manner the extracted proteins are white, powdery solids readily dissolving in water giving clear solutions. Reconstituted plasma can still be caused to clot and albumins may be crystallised.

The extracted serum proteins are dissolved in water and the volume made up to that of the original serum, and the euglobulin and pseudoglobulin removed in turn by additions of saturated ammonium sulphate solution to render the mixture one-third and one-half saturated with ammonium sulphate. The albumins are then either caused to crystallise or precipitated by addition of small amounts of 10 % acetic acid and saturated ammonium sulphate solution, the reaction being adjusted to p_H about 4·9. Precipitation of albumin by adjusting the reaction and addition of ammonium sulphate solution is preferable to addition of solid ammonium sulphate. The protein fractions are filtered off on Büchner funnels using Schleicher and Schüll hardened papers (No. 575), redissolved in water and reprecipitated twice more in a similar manner. This fractionation is sufficient for the present purpose. The proteins are then dissolved in water and dialysed in collodion membranes against distilled water in an ice-chest, the outer liquid being changed hourly for the first day and then daily for at least a week. The membranes used were tested for permeability to salts and dyes and were all impermeable to serum proteins. In the case of albumin and pseudoglobulin the dialysed solutions were used directly for the determination of rotatory power whilst the euglobulin was precipitated as a white solid on dialysis and was dissolved in 0·9 % sodium chloride solution. The solutions used were clear and colourless except the albumins which have a slight yellow colour in thick layers.

A Schmidt and Haentzsch polarimeter with a Hilger direct vision spectroscopic eyepiece was used for the measurement of rotations and a mercury

vapour lamp provided a light source for wave-lengths, 4358 (blue), 5461 (green), and 5780 (yellow) Ångström units, and a Nernst lamp with a crystal-violet filter, red light (6600 Å.U.). For the determination of proteins the author is indebted to Dr J. R. Marrack who used the micro-Kjeldahl method, using the factor 6·45 for the conversion of nitrogen content to protein concentration.

Serum albumin.

In this section all rotatory powers are given for the mercury green line at 20° ($[\alpha]^{20°}_{5461}$), rotations for this light being most accurately determined.

Table I. $[\alpha]^{20°}_{5461}$ *of serum albumins.*

	Crystalline			Precipitated				
	Horse 1	Horse 2	Horse 3	Horse 4	Ox 5	Human 6	Human 7	Mean
p_H 4·9	−78·4°	−81·0°	−78·7°	−76°	−79·6°	−79·3°	−77·7°	−78·1°
p_H 7·2	−78·4°	−81·5°	−81·8°	—	—	—	—	—

It is to be noted that:

(1) Precipitated and crystallised extracted serum albumins have approximately equal rotatory powers.

(2) Alteration of the p_H from 4·9 to 7·2 has but little effect on the rotation.

(3) Horse, ox, and human serum albumins have about the same rotatory powers.

(4) The results are in agreement with those of Young [1922], *i.e.* − 78·3° in place of − 78·1°, in contradistinction to those of Lewis [1922] and other workers who worked with proteins contaminated with lipins. Lewis used only the D-line for his determinations of rotatory power, but, on extrapolating his results on the basis of the curves obtained by us, his value for horse albumin is − 70°. Using unextracted serum albumin we have obtained similar results, but, as Lewis found, it is difficult to obtain concordant results with such material.

If the albumins are allowed to remain in contact with alcohol at the ordinary temperature, or with acidified ammonium sulphate solution for long periods, or if the protein solutions are heated, a certain amount of denaturation occurs and the rotatory power is increased. Samples 8, 9 and 10 in Table II and possibly 2 in Table I were partly denatured.

Table II. *Denatured albumins (horse serum).*

	8	9	10
	Crystalline	Precipitated	
$[\alpha]^{20°}_{5461}$	−90·0°	−89·0°	−84·3°

Serum pseudoglobulin.

Table III. $[\alpha]^{20°}_{5461}$ *of serum pseudoglobulin in water.*

Horse 1	Horse 2	Horse 3	Human 4	Ox 5	Mean value
−68·8°	−66·2°	−69°	−70·7°	−69°	−68·7°

Good agreement is obtained between the values of $[\alpha]_{5461}^{20°}$ obtained for extracted horse, human and ox serum pseudoglobulin. The mean value of $-68{\cdot}7°$ is higher than that calculated from Lewis's results for unextracted horse serum pseudoglobulin ($-62{\cdot}1°$).

Serum euglobulin.

Table IV. $[\alpha]_{5461}^{20°}$ *of extracted serum euglobulin.*

Horse		Ox	
1	2	3	Mean
$-60{\cdot}1°$	$-61{\cdot}3°$	$-62{\cdot}1°$	$-61{\cdot}2°$

Good agreement is obtained between the values of $[\alpha]_{5461}^{20°}$ for horse and ox extracted serum euglobulin and the values are higher than those calculated from Lewis's figure ($-51{\cdot}0°$) for unextracted horse euglobulin.

Egg-albumin.

To the whipped white of fresh eggs is added an equal volume of saturated ammonium sulphate, and after standing over night the precipitate is filtered off and the albumin in the filtrate precipitated by acidification and addition of ammonium sulphate. The precipitated albumins are filtered off, dissolved in water and extracted first with alcohol and ether in the cold, and then with ether at the ordinary temperature, and then dried, as described in the case of serum proteins. The extracted egg-albumin thus obtained gives a clear solution in water even at the isoelectric point and crystallises, but with some difficulty, in the ordinary way. Sørensen [1926] expresses the opinion that egg-albumin cannot be extracted with alcohol and ether in the cold without denaturation, and takes as criteria of undenatured egg-albumin, solubility in water and crystallisability. The extracted protein is dissolved in water, the solution made neutral, an equal volume of saturated ammonium sulphate is added, the solution filtered and the egg-albumin twice precipitated by additions of appropriate amounts of 10 % acid and saturated ammonium sulphate solution. The egg-albumin thus obtained is dissolved in water and dialysed until free from salts in the usual way. The albumin contained 7·7 mg. of phosphorus per 1 g. of nitrogen which corresponds to a molecular weight for egg-albumin of about 26,000, assuming there is 1 atom of phosphorus per molecule of protein. The generally accepted values for the molecular weight lie between 27,000 and 34,000 [cf. Cohn, Prentiss and Hendry, 1925]. The specific rotatory power, $[\alpha]_{5461}^{20°} - 44{\cdot}5°$, is higher than that obtained by Young [1922] for unextracted egg-albumin ($-37{\cdot}5°$).

Lactalbumin.

The caseinogen is removed from fresh skimmed milk either by addition of dilute acetic acid under the surface with vigorous stirring or by treatment at 37° with rennin for 45 minutes. The precipitated casein is removed by

filtration, and the lactalbumin is precipitated by addition of dilute acetic acid until the solution has p_H about 4·9, and of saturated ammonium sulphate solution. After standing, the precipitated proteins are filtered off, dissolved in water and extracted with a mixture of alcohol and ether in the cold and with ether in a Soxhlet apparatus, and dried *in vacuo* in the usual way. The white powdery protein is dissolved in water, the solution made neutral, an equal volume of saturated ammonium sulphate added, and the solution filtered. The lactalbumin is then twice precipitated by addition of dilute acetic acid and saturated ammonium sulphate solution, and filtered on Büchner funnels. The purified lactalbumin is then dissolved in water and dialysed until free from salts. The rotatory power is $[\alpha]^{20°}_{5461} - 47·5°$. The phosphorus content is 5·4 mg. per 1 g. of nitrogen in the protein. The molecular weight of lactalbumin, assuming that there is 1 atom of phosphorus in each molecule of lactalbumin, is thus about 37,000. For the phosphorus determinations the author is indebted to Dr Kay.

Caseinogen.

Caseinogen was prepared from milk by the method of Van Slyke and Baker [1918] as modified by Cohn [1922].

Gelatin.

Isoelectric gelatin was prepared from Coignet's "Gold Label" gelatin by the method of Loeb [1922].

Table V. *Specific rotatory powers of proteins and tyrosine for light of different wave-lengths.*

	$[\alpha]^{20}_{\lambda}$			
Protein	λ 4359	λ 5461	λ 5780	λ 6660 Å.U.
Serum albumin	−151·5°	−78·1°	−67·0°	−47·3°
Serum pseudoglobulin	−118·2	−68·7	−59·8	−43·1
Serum euglobulin	−105·2	−61·2	−54·0	−39·4
Egg-albumin	−83·9	−44·5	−38·3	−27·5
Lactalbumin	−88·9	−47·5	−41·1	−28·6
Caseinogen	−184·4	−105·1	−91·2	−66·0
Gelatin	−496·8	−282·8	−248·2	−181·1
Tyrosine in 20 % HCl	−7·8	−9·6	−9·3	−7·2
„ 4 % HCl	−14·4	−12·5	−11·5	−8·9
„ 4 % NaOH	−22·9	−15·1	−13·1	−9·8
„ 20 % NaOH	−27·8	−17·4	−15·2	−11·2

In Table V are given the specific rotatory powers of the proteins examined for light of different wave-lengths. To confine attention for the moment to the rotation for the mercury green line ($\lambda = 5461$ Å.U.), which is probably most accurately determined, the following conclusions may be drawn.

(i) The considerably greater (10–15 %) rotation of lipin-free proteins, as pointed out in the experimental section.

(ii) The marked difference between the rotation of serum albumin and globulins, and between serum pseudoglobulin and euglobulin.

(iii) Caseinogen has a high laevo-rotation, $[\alpha]^{20°}_{5461} - 105°$, not a dextro-rotation as is inferred from the work of Zaykowksy [1923]. Tyrosine is included to show the effect of p_H on dispersion. This will be referred to later.

Optical rotatory dispersion.

It is well known that, in general, optical rotation increases with decreasing wave-length of the light used for the determination; but Drude in a treatise on optics in 1907 was the first to obtain a satisfactory mathematical expression connecting rotatory power and wave-length of the light.

Drude's equation is:

$$\alpha_\lambda = \frac{k_0}{\lambda^2 - \lambda_0^2} \pm \frac{k_1}{\lambda^2 - \lambda_1^2} \ldots \text{etc.},$$

where α_λ = rotation for light of wave-length λ,
k_0, k_1 = constants,
λ_0, λ_1 = constants.

The rotation may be controlled by one, two, or more terms in the Drude equation. λ_0, λ_1 etc., have a further significance in that they are the characteristic wave-lengths of oscillating electrons in the molecule and as such give rise to bands in the absorption spectrum of the substance, so that a direct relationship exists between the rotatory dispersion and the absorption spectrum of the substance. Lowry [1913] was among the first to apply the Drude equation to chemical substances. He showed that if the rotatory powers of a substance can be expressed, as those of most substances can, by a single term Drude equation, *i.e.* $\alpha_\lambda = \frac{k_0}{\lambda^2 - \lambda_0^2}$, then a linear relationship exists between the reciprocals of the rotatory powers $\left(\frac{1}{\alpha_\lambda}\right)$ and the squares of the wave-lengths (λ^2). This follows from a rearrangement of the equation:

$$\frac{1}{\alpha_\lambda} = \frac{\lambda^2}{k_0} - \frac{\lambda_0^2}{k_0};$$

and if $\frac{1}{\alpha_\lambda} = 0$, then $\lambda = \lambda_0$. That is to say, if $\frac{1}{\alpha_\lambda}$ is plotted against λ^2 the point where the line cuts the zero axis will indicate the wave-length of the absorption band controlling the dispersion.

In Fig. 1 are plotted the dispersions of the proteins in the way described. No deviation from a linear relationship between $\frac{1}{\alpha_\lambda}$ and λ^2 in the visible region of the spectrum can be detected.

Serum albumin, egg-albumin and lactalbumin all have similar dispersions, the dispersion curve cutting the axis at a point at, or near to, $\lambda = 2760$ Å.U., which is the wave-length of the head of the absorption band in serum proteins nearest to the visible portion of the spectrum. In serum pseudoglobulin, euglobulin and caseinogen, however, the line appears to strike the axis at a point nearer the region of shorter wave-length, although these proteins have similar absorption spectra to the albumins. It would thus seem that in the albumins the absorption band of longest wave-length dominates the dispersion, whilst in the other proteins other bands have a predominant effect.

The case of gelatin is interesting since, owing to its low content of amino-acids containing a benzene nucleus, it does not possess absorption bands as near the visible region of the spectrum as the other proteins, and it is found, as expected, that the dispersion is correspondingly modified.

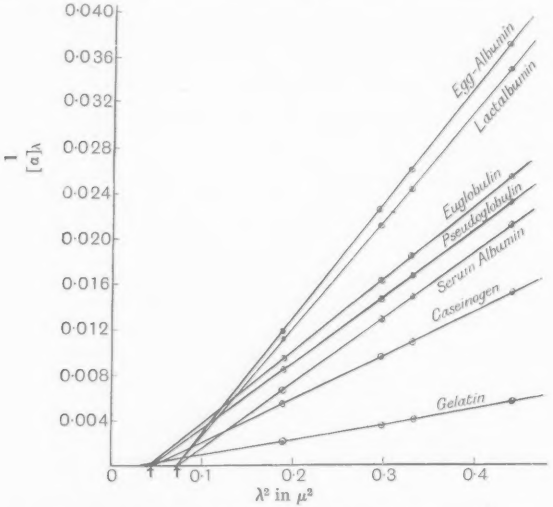

Fig. 1. Dispersion curves of proteins. Relation between $\frac{1}{[\alpha]_\lambda}$ and λ^2.

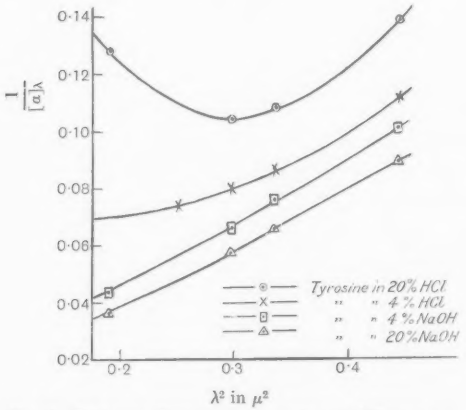

Fig. 2. Rotatory dispersion of tyrosine at different p_H.

The optical rotatory dispersion of tyrosine is more complex than that of the proteins, that is to say, two or more terms of the Drude equation are required to express the rotations for different wave-lengths (see Fig. 2).

Hydrogen ion concentration has a marked effect on the dispersion. In tyrosine therefore several absorption bands have a great influence on the rotation. Tyrosine has a very similar absorption spectrum to that of serum proteins [Stenström and Reinhard, 1925], and it does not vary greatly with alteration of p_H. In two respects therefore the absorption spectra and rotatory dispersion vary. The absorption spectra of serum proteins and of tyrosine are very similar but the rotatory dispersion is different; and the absorption spectrum of tyrosine is not greatly affected by hydrogen ion concentration whilst the rotatory dispersion is altered by change of p_H.

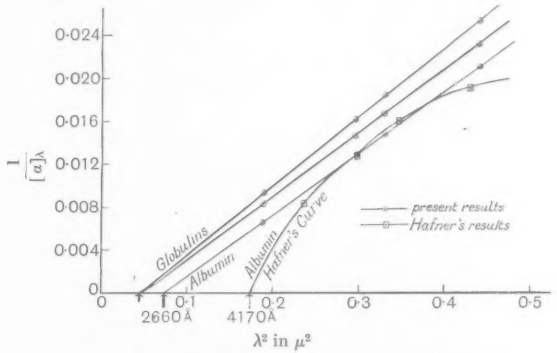

Fig. 3. Dispersion of serum proteins showing Hafner's [1925] results.

Hafner's results [1925] differ markedly from ours. First he finds the rotations of globulins higher than those of albumins, which is not supported by the work of other authors. In Fig. 3 are plotted Hafner's dispersion of serum albumin and our results on serum proteins. Hafner's figures (which are plotted inaccurately in his paper) lead him to the conclusion that the characteristic wave-length of an oscillating electron in the albumin molecule (*i.e.* that of an absorption band) is 4170 Å.U.; this would involve an absorption band in the visible region of the spectrum, an entirely unconfirmed hypothesis. Also, by carrying the determinations of rotatory power further into the blue region of the spectrum, we have shown Hafner's results to be inaccurate. Deviations of Hafner's curve from the straight line obtained by us are readily explicable on the basis of experimental errors, due to lack of duplicate determinations, dilution of solutions, etc. on Hafner's part.

Physical properties of the globulins.

Chick [1914] and other workers express the opinion that euglobulin is a mechanical complex of pseudoglobulin and lipins which can be dissociated by extraction of the lipins with alcohol and ether. We have been unable to obtain evidence of such separation. After extraction with alcohol and ether

at − 4° and with ether at laboratory temperature for 24 hours, euglobulin and pseudoglobulin retain their characteristic individual properties.

	Extracted euglobulin	Extracted pseudoglobulin
Solubility in saline	Soluble	Soluble
Solubility in distilled water	Insoluble	Soluble
When dialysed against distilled water	Precipitated	Remains in solution
$[\alpha]^{20°}_{5461}$	−61·2°	−68·7°

Summary.

1. The rotatory powers of lipin-free serum albumin, pseudoglobulin and euglobulin, lactalbumin, egg-albumin, caseinogen and gelatin for different wave-lengths have been determined.

2. Extracted serum albumin, pseudoglobulin and euglobulin have different and distinct rotatory powers. Euglobulin is not a mechanical complex of pseudoglobulin and lipin.

3. Serum albumin, egg-albumin and lactalbumin have rotatory dispersions dominated by an absorption band at about 2760 Ångström units. Serum globulins, caseinogen, and gelatin have different rotatory dispersions from the albumins.

4. Extraction of lipins from proteins results in a considerable increase of rotatory power, but crystallised and non-crystalline serum albumins have practically identical properties.

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References.

Chick (1914). *Biochem. J.* **8**, 404.
Cohn (1922). *J. Gen. Physiol.* **4**, 697.
Cohn, Prentiss and Hendry (1925). *J. Biol. Chem.* **63**, 720.
Fredericq (1880). *Arch. Biol.* **1**, 47.
Gürber (1894). *Sitz. Würzburger physiol.-med. Ges.* 13.
Hafner (1925). *Biochem. Z.* **166**, 424.
Hardy and Gardiner (1910). *J. Physiol.* **40**; *Proc.* lxviii.
Hartley (1925). *Brit. J. Exp. Path.* **6**, 180.
Lewis (1922). *Proc. Roy. Soc. Lond.* B, **93**, 178.
Loeb (1922). Proteins and Theory of Colloidal Behaviour (New York and London), p. 35.
Lowry (1913). *J. Chem. Soc.* **103**, 1062.
Maximovitsch (1901). *Maly's Jahresber*, **31**, 35.
Michel (1895). *Sitz. Würzburger physiol.-med. Ges.* **29**, 117.
Porges and Spiro (1902). *Hofmeister's Beiträge*, **3**, 277.
Reiss (1904). *Hofmeister's Beiträge*, **4**, 150.
Sebilin (1885). *Z. physiol. Chem.* **9**, 445.
Seng (1899). *Z. Hyg. Infektionskrank.* **31**, 513.
Van Slyke and Baker (1918). *J. Biol. Chem.* **35**, 127.
Sørensen (1926). *J. Chem. Soc.* 3007.
Starke (1881). *Maly's Jahresber.* **11**, 17.
Stenström and Reinhard (1925). *J. Phys. Chem.* **29**, 1477.
Young (1922). *Proc. Roy. Soc. Lond.* B, **93**, 15.
Zaykowsky (1923). *Biochem. Z.* **137**, 502.

XXXII. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

I. PREPARATION AND GENERAL CHARACTERS.

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In a previous paper [Chibnall and Grover, 1926], there has been described the preparation in bulk of the cytoplasm from green leaves; in that paper it was shown that the cytoplasm consists chiefly of proteins and substances soluble in fat solvents and the methods of preparation and the properties of these proteins were discussed. As part of this cytoplasmic protein appeared to be in some state of physical or chemical combination with substances of fatty nature, it was deemed wise to attempt to study the nature of the ether-soluble substances of cytoplasm before embarking on more advanced studies of the proteins themselves.

A review of the existing literature at once discloses the rather surprising fact that although numerous references to the isolation of individual substances of fatty nature abound, no thorough investigation of the fatty materials present in the leaf has been attempted. This is probably due to the fact that the extraction of the fatty material from fresh leaves—in which both the cell wall and cytoplasm are heavily imbibed with water—is attended with considerable difficulties. In such work on the plant as has been reported, the seed or young seedlings appear to have been studied chiefly, and the method usually adopted has been to extract with boiling alcohol, evaporate the extract to dryness and to treat the residue obtained with ether. This method cannot be satisfactory when applied to fresh leaf material in bulk. In the first place, the extraction of say 20 kg. of fresh leaves would require a prohibitive amount of alcohol; secondly, the dilution of the alcohol by the water in the leaf is sufficient to cause the solution of a certain amount of the sugars, water-soluble nitrogenous substances and inorganic salts which have to be subsequently removed; thirdly, substances insoluble in alcohol but soluble in ether may be present, and this entails subsequent exhaustive extraction of the bulky leaf material with ether.

Alternatively the leaf material has been dried and ground to a very fine

powder. Although this process results in a much less bulky product and one which can be directly treated with fat solvents, there are objections to it, for the drying of large quantities of leaf material is necessarily slow, with the resulting danger of considerable alteration of the substances present due to enzyme action to an extent which we have no means of judging. Further the continual grinding, which must be carried out if the material is to be reduced to that very fine state of division which is so necessary for successful extraction, may result in a considerable degree of oxidation.

Even if the difficulties of dealing with moderately large quantities of material are overcome, there still remains the fact that the yield of fatty substances from green leaves is, in general, very small in terms of the weight of the fresh leaf taken, even though the fat constitutes no inconsiderable proportion of the total leaf solids. Thus during the investigation reported in this paper, upwards of 200 kg. of fresh leaves have been used, and yet as will be seen later, we have been limited throughout the work for want of sufficient fatty material. These considerations are probably sufficient to explain the paucity of information available on the chemistry of the leaf fats.

The problem of obtaining sufficient material is thus one of devising means whereby very large quantities of leaf material can be reduced rapidly to small bulk in a form which can be directly extracted with fat solvents without the necessity of preliminary drying.

General Experimental Methods.

The present paper deals not with the ether-soluble substances of the total leaf but only with those which appear to be part of the colloidal complex which constitutes the cytoplasm of the leaf cells. In previous papers methods of obtaining this cytoplasm free from cell-wall material, and the preparation of proteins from it have been described [Chibnall and Grover, 1926]. In those papers it was considered justifiable to give to these proteins the generic name of cytoplasmic proteins. This terminology neglects the nucleus. However, in the mature leaf-cell the mass of the nucleus is very small compared to that of the cytoplasm. Therefore it may be well if we emphasise here the fact that the proteins were obtained in such quantity as to make it quite definite that they must be derived in bulk from the cytoplasm itself and not from the nucleus. Similarly in the present work we have found no single ether-soluble substance which can definitely be assigned to the nucleus, for the methods available for the isolation of the individual constituents of any complex fatty material are such that unless a very large quantity can be used it is a matter of considerable difficulty to obtain them in a pure state unless they are present to the extent of 5 % or more. We have thus been compelled to confine our attention to those constituents which are present in amounts too large for them to be present in the nucleus only, and therefore just as in the work on the proteins, we may regard the ether-soluble substances with which we are dealing as forming part of the cytoplasm.

The aim in this work has been to study as quantitatively as possible the ether-soluble extractives of the cytoplasm of one leaf only in order that the knowledge so obtained may be of possible use later in a similar study with small preparations from different species of leaves. With small quantities of material, little can be done save to determine the usual fat constants, and the results of the investigation into a larger quantity of similar fat will help to make the interpretation of those constants more of a probability and less of a gamble. The green leaves of unheaded cabbage—*Brassica oleracea*—were chosen for this work because they can be obtained throughout the year. Further, in this initial study, it has been desired to enquire in greater detail into the nature of the phosphorus-containing substances soluble in ether, and as preliminary investigation into the fats of a number of species of leaves seemed to show that the cabbage leaf was richer in ether-soluble phosphorus than the leaves of the other species which were used, that leaf was finally chosen.

Details of preparation.

After the separation of the leaves and the removal of their thick mid-ribs, the material was coarsely minced, mixed with an equal volume of distilled water, then finely minced and finally squeezed through fine silk. The residue of cellular material, after being treated with water, was again minced and squeezed out as before. Previous experimental work has shown that this deep green solution contains most of the vacuole constituents and part of the cytoplasm, the latter being dispersed in colloidal form, and that it does not contain any cell-wall material. The green juice is then heated to 70° when the cytoplasmic material is coagulated, and on cooling as rapidly as possible it settles as a green amorphous mass, leaving a clear brown fluid which is easily filtered through folded filters. The coagulated cytoplasm was enveloped in stout filter cloth, placed in a Buchner press and submitted to a pressure which was slowly increased to 400 kg. per sq. cm. and maintained until no more liquid was expressed. The product thus obtained was a dark green hard mass which could be readily powdered in a mortar in spite of the fact that analysis showed that it still contained about 40 % water. Such material is in a form which can be directly extracted with ether.

Prepared by this method the crude mass of cytoplasmic material will contain also those substances present in the vacuole fluid which are precipitable by heat. Previous experimental work [Chibnall, 1923] has shown that these are present in relatively small amounts, and consist of protein and inorganic material. Consequently, as the ether-soluble substances of the cytoplasm and not the proteins are now under investigation, these latter may be disregarded. Further, water-soluble substances from the vacuole will be present in the water still retained by the preparation, but, since the final solvent used for extraction of the fat is anhydrous ether, the presence of these substances will not affect the composition of the material obtained. A more

refined method of preparing cytoplasm has been already described [Chibnall and Grover, 1926], but on account of its more lengthy nature it could not be used in the present research. The essential point was to obtain the ether-soluble substances of the cytoplasm in large quantities, and the presence of vacuole protein and salts will not affect the question.

The material was extracted with ether in a Soxhlet apparatus with frequent changing of the receiving flasks, until the final extract was virtually colourless.

It was considered wise to make a study of the rate at which the fat was removed by ether, and the following table shows the weights of fat obtained during the various stages of a typical extraction.

Table I. *Showing the rate of extraction of the ether-soluble substances from 149 g. of cabbage leaf cytoplasm.*

Batch F, 9·5 kg. of leaves picked May 20, 1926.

Number of extract	Duration of extraction hours	Weight of extract g.
1	16	17·0
2	24	0·6
3	24	0·15
After regrinding of the powder:		
4	24	0·23
5	24	0·02

Schulze and Steiger [1889] reported that the phospholipins can only be completely removed from seed material if the ether extraction is followed by successive extractions with alcohol at 60°. It became of interest to see whether this finding applied also to the cytoplasmic phospholipins of the leaf. Accordingly after the exhaustive extraction with ether tabulated above, the residue was treated three times with large volumes of alcohol maintained at 60° and renewed every half hour. The combined alcohol extracts were evaporated to dryness at low pressure, and the residue treated with anhydrous ether. The total weight of this extract was 0·88 g. Finally, to ensure that there was no appreciable amount of fatty material left in the residual powder, the whole of the latter was hydrolysed for 3 hours with *N* HCl on a boiling water-bath. The mass was filtered and the residue washed several times with alcohol and then with ether. The alcohol-ether washings were combined with the ether extract of the filtrate and after thorough washing with water to remove alcohol, were evaporated to dryness. The weight of fatty acid so obtained was 0·69 g.

It will be seen that the extraction by ether is virtually completed at the end of 40 hours, when an extract weighing 17·6 g. has been obtained. Subsequent treatment of the residue with alcohol yielded 0·88 g. and hydrolysis 0·69 g. We therefore adopted ether extraction for 40 hours as our routine method, and the relatively small amount of material which can be obtained by subsequent treatment with alcohol and by hydrolysis has been ignored in this paper. It may be mentioned in passing that the alcohol extract does

not yield any quantity of phospholipin as Schulze showed to be the case in seed material.

The ether extracts contained a little water which had been removed by the ether from the coagulum. It has been our experience that the presence of water in such a solvent as ether will allow a small but definite amount of inorganic material and amino-compounds to go into solution. Since subsequent work on the extract was carried out with a view to determining the nature of the phosphorus-containing substances present, and since the percentage of nitrogen in those bodies is very low, it was considered essential to evaporate every crude extract to dryness *in vacuo* and make an anhydrous ether extract from it[1]. There always remained behind a small amount of material undissolved by that solvent, and this material was rejected.

Preliminary analysis of the extracted cytoplasm.

Tables II and III give details of the various samples of cytoplasm prepared. The method employed for the preparation of the cytoplasm in bulk is such that much of the cytoplasm is left behind in the cell residues. The amount actually obtained is dependent on the thoroughness with which the leaves were ground: only those cells which have been torn open will have their cytoplasm dispersed into colloidal solution. Some information may be obtained regarding the percentage of cytoplasm extracted by a determination of the protein content of the original leaf and of the cytoplasmic coagulum respectively. If such a rough method of calculation be adopted, it is only with the knowledge that it may involve an error of 5 %.

Table II. *Details of cytoplasm prepared from green leaves of cabbage.*

(Batch E, 20 kg. of leaves picked April, 1926.)

Total leaf solids	2550 g.
,, N	67 g.
,, water-soluble N	23·9 g.
,, protein-N	43·1 g.
,, protein (N × 6·25)	269·3 g.
,, cytoplasm (calculated)	405·1 g.
Percentage of total cytoplasm extracted	47 %

Extracted cytoplasm	g.	% total leaf solids
Protein (N × 6·25)	129	5·73
Ether-soluble substances	39	1·73
Ash	12	0·53
Undetermined	14	0·62
	194	8·61

From Table III it will be seen that the percentages of protein and ether-soluble substances in the various preparations are very constant for biological material. Further the protein : ether-soluble substances ratio is always about 3 : 1. These concordant figures are good evidence that the samples of material with which we are dealing are truly representative of the cytoplasm as a

[1] In this and subsequent papers the expression anhydrous ether means "0·720 ether" dehydrated by sodium.

whole. Thus, to consider extreme cases, in batches D and H, where the percentage of cytoplasm extracted was 41 and 28 respectively, the ratio is the same. Further evidence as to the uniformity of the different samples will be obtained by a study of the figures in Table IV which deal with the composition of the ether-soluble substances.

Table III. *Analyses of cytoplasm prepared from several different batches of green leaves of cabbage.*

	Autumn sown picked April and May				Spring sown picked August	
	D	E	F*	G	H	M
Weight of fresh leaves, kg.	20	20	9·5	9·0	32	50
	g.	g.	g.	g.	g.	g.
Weight of cytoplasm extracted	167·6	194·4	79·6	70·6	194·0	389·2
Protein (N × 6·25)	110·3	129·0	50·9	44·1	122·0	223·6
Ether-soluble substances	35·4	39·0	18·0	14·2	40·0	75·4
Ash	9·5	12·4	2·7	3·8	14·2	40·7
Undetermined	12·4	14·0	8·0	8·5	17·8	49·5
Ratio protein/ether-soluble substances	3·1	3·3	2·83	3·1	3·1	3·0
Composition of cytoplasm:	%	%	%	%	%	%
Protein (N × 6·25)	65·8	66·4	63·9	62·4	62·9	57·2
Ether-soluble substances	21·8	20·1	22·6	20·1	20·6	19·3
Ash	5·7	6·4	3·4	5·4	7·3	10·5
Undetermined	7·4	7·1	10·1	10·1	9·2	13·0
Percentage of total cytoplasm extracted	41	47	39	36	28	32

* Exhaustive ether extraction.

A point worthy of notice is that the ether-soluble substances constitute one-fifth by weight of the total cytoplasmic solids. The ether extract on evaporation to dryness yielded a solid fat deeply pigmented with chlorophyll. We should have preferred to have removed the chlorophyll from the coagulum before the extraction of the fat. Since it was desired to obtain the fat in a state as little altered as possible from that in which it occurred in the leaf, the use of chemical methods of separating the chlorophyll was rendered impossible. Though many attempts were made to extract the pigment by the use of solvents, these proved fruitless, because all those solvents which will remove chlorophyll will dissolve some part of the fat. Therefore, in order to keep the work as quantitative as possible, the chlorophyll and fat were weighed together. The presence of this chlorophyll has necessarily modified the constants which would have been obtained had the fat been chlorophyll-free. Evidence will be adduced in a later paper, however, to show that the difficulties caused by the presence of the pigment can be overcome, and an approximate estimate of its amount and its effect on various determinations made.

The figures given in Table IV define the general characteristics of the ether-soluble substances. The percentage of material given under I was calculated by weighing the precipitate obtained when a solution of these substances in

dry ether was treated with 4 volumes of acetone. This high percentage figure for acetone-insoluble material does not represent phospholipins, as might be expected. It will be shown subsequently that not more than half of the weight of this fraction is due to substances containing phosphorus, and that these substances are nitrogen-free and therefore not phospholipins in the strict chemical sense. The other components of this fraction, which do not contain phosphorus, consist of one or more hydrocarbons and alcohols. The acetone treatment was found to remove all the phosphorus-containing substances and the figures given for the percentage of phosphorus-free material in II are obtained by difference.

The filtrate from I was evaporated to dryness *in vacuo*; on this material determinations of iodine value (Wijs) and saponification value were carried out. Attention is drawn to the very high iodine value and to the fact that the low saponification value is due not to the presence of fatty acids of very high molecular weight, but to the large percentage of unsaponifiable matter.

Table IV. *Showing the general characters of the ether-soluble substances of the cytoplasm.*

(All weights given are percentages of total ether extractives.)

	D	E	F	K
I. Weight of material precipitated by acetone	41·5	42·0	41·0	44·0
II. Weight of phosphorus-free fatty material	48·5	58·0	59·0	56·0
Iodine value (Wijs)	141	147	152	154
Saponification value	151	158	152	149
III. Weight of unsaponifiable matter	16·9	16·5	17·0	24·1
Iodine value (Wijs)	113	117	119	105
Weight of sterol	3·63	2·65	3·70	3·80
IV. Weight of fatty acids	23·2	23·1	23·0	15·0
Iodine value (Wijs)	173·5	203·5	201	206
Neutralisation value	209	206	198	197

The unsaponifiable matter was determined by the method already described [Drummond, Channon and Coward, 1925], the second saponification being carried out with sodium ethylate in alcohol, which is particularly necessary in dealing with plant materials containing definite quantities of the esters of the higher alcohols. The sterol content was determined by the digitonin method [Windaus, 1910].

The fatty acids were prepared after the removal of the unsaponifiable matter and were rendered free from chlorophyll degradation products.

The figures given in Table IV indicate how very constant the various values obtained from different batches of materials are. This confirms the results recorded in the previous tables and suggests that the fraction of cytoplasm obtainable is quite representative of the whole.

SUMMARY.

The difficulties of preparing the ether-soluble substances of leaves are discussed. A method is given for preparing in bulk the ether-soluble substances of leaf cytoplasm, and the fat constants are given for several preparations made from cabbage leaves. The general uniformity of the results obtained is taken as evidence that the portion of cytoplasm extracted for analysis is representative of the whole.

REFERENCES.

Chibnall (1923). *J. Biol. Chem.* **55**, 333.
Chibnall and Grover (1926). *Biochem. J.* **20**, 108.
Drummond, Channon and Coward (1925). *Biochem. J.* **19**, 1047.
Schutze and Steiger (1889). *Z. physiol. Chem.* **13**, 365.
Windaus (1910). *Z. physiol. Chem.* **65**, 110.

XXXIII. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

II. CALCIUM SALTS OF GLYCERIDEPHOSPHORIC ACIDS.

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Introduction.

In the previous paper the methods for preparing the ether-soluble substances of the cytoplasm from leaf cells on a large scale were described, and an account given of the general nature of the substances obtained from the green leaves of unheaded cabbage.

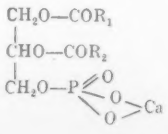
Determinations of the phosphorus content of the fatty material showed that this element is present in varying amounts. Thus batches *E*, *F*, *G* and *H* contained respectively 1·0, 1·6, 1·89, 1·1 % of phosphorus. It was mentioned in that communication that if four volumes of acetone be added to the solution of the ether-soluble substances in anhydrous ether in order to remove the phospholipin fraction, a large proportion of the material was precipitated. Thus from different preparations, 41, 42, 41 and 44 % of the ether-soluble material was precipitated in this way. Although the amount of phosphorus in the batches varied considerably, it will be seen that the amount of precipitate obtained by the addition of excess of acetone was constant. Now since the phosphorus was found to be almost quantitatively removed from the ether solution by precipitation with acetone, it follows that the phosphorus content of the acetone precipitate must vary in proportion to the phosphorus content of the ether solution from which it is derived. If we assume, therefore, that only one compound containing phosphorus is present in the ether solution, it follows that the acetone precipitate must necessarily contain at least one substance which is phosphorus-free. Actually it will be shown that there are present in it at least one solid hydrocarbon and one higher alcohol, both of which can be removed by treatment with boiling acetone or alcohol and subsequent filtration. It is thus apparent that the constant percentage of the ether-soluble substances precipitated from dry ether solution by the

acetone is fortuitous, because this precipitate is made up of at least three substances which happen to be present in varying amounts, but the sum total of which is constant.

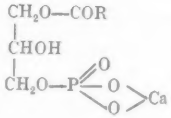
This communication deals only with the material which remains undissolved when the precipitate obtained by adding excess of acetone to the ether solution has been exhaustively extracted with boiling acetone. This fraction which is insoluble in hot acetone contains virtually all the phosphorus of the original ether extract; further every preparation made from different batches of leaves had a similar elementary composition. Thus the phosphorus content of the material from batches *D*, *F* and *H* was 4·9, 4·86 and 4·59 %; they contained 0·22, 0·23 and 0·22 % of nitrogen.

The appearance of this material, its rapid oxidation in air and the fact that it yielded about 70 % of its weight of fatty acids on hydrolysis, made it appear probable that it was of phospholipin character. Against this view however was the fact that its nitrogen content was very low, and it was certain that if this nitrogen were represented by lecithin, the bulk of the material must be of a different nature, as 0·2 % of nitrogen will only account for 10 % of the phosphorus present if calculated on the basis of lecithin. The possibility of the material being a uniform substance with an N : P ratio of 1 : 10 was considered highly improbable. It was therefore argued that this mixture probably contained two substances, one containing nitrogen and possibly phosphorus, but the bulk made up of a substance containing over 4 % of phosphorus and being nitrogen-free. If the molecule of the latter substance were built up in any way similarly to what we accept as the structure of lecithin, there must be some grouping to replace the choline radicle and the grouping must be nitrogen-free. The probability of it being a sugar was negatived, because hydrolysis with methyl alcoholic HCl gave no products which would reduce an alkaline copper solution; nor were any carbohydrate reactions obtained. It seemed probable, therefore, that a metal was replacing choline. The likely metals are K, Mg, Na, and Ca; and the last was the one actually found to be present in the molecule. Further, determinations of the calcium and phosphorus present, showed that in every preparation made, these substances bore an atomic ratio in all cases very close to unity.

Since the second substance has a phosphorus content of about 4 %, and a calcium content corresponding to the same number of atoms of that element as of phosphorus, and since the percentage of fatty acids obtained on hydrolysis was very similar to that which such a body as lecithin would yield, we based our work on the hypothesis that it was the calcium salt of a diglyceride-phosphoric acid of the type



The analyses quoted later show that the crude calcium salt is a mixture of at least two substances, one the calcium salt of the diglyceridephosphoric acid just outlined, the presence and structure of which has been definitely established, and the other probably the calcium salt of a monoglyceridephosphoric acid of the type



At least three-quarters of the material insoluble in boiling acetone consists of the calcium salt of the diglyceridephosphoric acid: it is for this reason that we have made the preparation and analysis of the free diglyceridephosphoric acid the main thesis of this paper. The possible presence of the monoglyceridephosphoric acid and the absence of lecithin are discussed in two appendices.

In discussing the analytical data which follow, it will be simpler if the figures required by the calcium salt of distearylglycerophosphoric acid are taken as the theoretical basis. This is not strictly correct, for as appears later the fatty acids present in the molecule are mainly unsaturated; and hence the values required by theory will not be in strict accord with those obtained experimentally. Since a number of fatty acids seem to be present, and the crude calcium salt is thus really a mixture of substances containing different fatty acids, there is no alternative but to adopt some basis for theoretical calculation, and figures for the calcium salt of distearylglycerophosphoric acid have therefore been used.

Isolation of the crude calcium salt.

Batch H. Total weight of ether-soluble substances 62·3 g. : P 1·10 %; Ca 1·43 %. Ratio Ca to P is approximately 1:1.

Fraction precipitable by acetone. The fat was dissolved in 400 cc. of anhydrous ether and 1600 cc. of pure acetone added; the solution was left for 1 hour on ice and filtered. The residue was ground with acetone and refiltered. The mother-liquor was reduced *in vacuo* to a volume of 200 cc. and four volumes of acetone were again added and, after standing on ice overnight, the solution was filtered. The combined precipitates, which weighed 28·1 g., were redissolved in ether and again precipitated with acetone. Experiments with earlier samples had shown that repetition of this procedure, although reducing the weight of material finally obtained, makes no appreciable difference to its phosphorus content. In other words, both the phosphorus-containing substance and the other substances present have similar solubilities in the ether-acetone mixture. Another method of purification was thus necessary, and

finally exhaustive extraction with boiling acetone was adopted. The substances extracted by this hot solvent crystallise as a pale green powder as soon as the temperature of the filtrate falls to 40°. The acetone extraction of this 28·1 g. was continued therefore until no crystalline material appeared on cooling the filtrate. (The filtrate will always go cloudy on cooling even though all the crystalline substances have been removed, for the phosphorus compound has a definite, though small, solubility in the hot solvent.)

By this procedure two fractions were obtained.

(1) The fraction insoluble in hot acetone. This fraction contains the calcium and the phosphorus. Weight 11·46 g. (18·6 % of total ether-soluble substance).

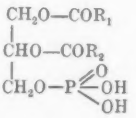
(2) The fraction soluble in boiling acetone. This consists chiefly of one or more hydrocarbons and alcohols, contaminated with traces of (1). The weight of this crystalline material was 13·0 g. (21 % of total ether-soluble substance); its nature will not be discussed further here, but will form the subject of a later communication.

Crude calcium salt H. The fraction insoluble in hot acetone, which we shall call the crude calcium salt, gave the following analysis:

0·0727 g. substance : 0·1524 g. CO_2, 0·0572 g. H_2O, 0·0142 g. ash.
0·4316 g. ,, 0·0712 g. $Mg_2P_2O_7$.
0·4316 g. ,, 0·0862 g. $CaSO_4$ and 0·0065 g. Fe_2O_3.
0·0888 g. ,, required (micro-Kjeldahl) 1·60 cc. 0·01 *N* acid.

	C	H	Ca	P	Ash	Fe	N
Found	57·16	8·74	5·88	4·59	19·54	1·08	0·25
Theory	63·10	10·10	5·39	4·18	17·20	0·0	0·0

It will be seen that although the calcium and phosphorus are present in the ratio 1:1 the values obtained for these elements are too high for the calcium salt of a saturated diglyceridephosphoric acid (containing two C_{18} acids), and, accepting the existence of the latter, it is to be surmised that a second substance containing calcium and phosphorus is also present. A substance of the type postulated above will readily lose its calcium when shaken in ether solution with aqueous mineral acid; there will then be produced an acidic substance of the type



which is a diglyceridephosphoric acid and which we may term the free acid.

With a view to obtaining some information regarding the possible second substance in the crude calcium salt a portion of about 5 g. of the latter was dissolved in ether, shaken with dilute HCl several times and the ether solution was well washed with water. By this procedure there were obtained 3·62 g. of free acid.

Crude free acid H:

0·3625 g. substance : 0·0505 g. $Mg_2P_2O_7$.
0·0727 g. „ required (micro-Kjeldahl) 1·50 cc. 0·01 *N* acid.

	P	N
Found	3·87	0·29
Theory	4·40	0·0

These figures show that a large proportion of the phosphorus has become water-soluble and been removed. The significance of this, together with the reasons for the phosphorus value being so low, will be discussed later. 0·727 g. of this free acid in ether solution was shaken out several times with lime water and the ether solution washed, reduced to a volume of 10 cc., and four volumes of acetone added to precipitate the regenerated calcium salt.

Regenerated crude calcium salt H:

0·1340 g. substance : 0·0184 g. $Mg_2P_2O_7$.
0·1340 g. „ 0·0265 g. $CaSO_4$.

	P	Ca
Found	3·83	5·82
Theory	4·18	5·39

It will be seen from these figures that the calcium : phosphorus ratio is now 1 : 1·18, in other words excess of calcium is present. This high value for calcium suggested that some other calcium-phosphorus-fatty acid compound in the original crude salt had broken down on being shaken out with acid. As a result the phosphorus has passed into the aqueous layer and been lost, whereas the fatty acid has remained in the ether layer along with the diglyceridephosphoric acid. On conversion of the latter into the calcium salt this contaminating fatty acid has been converted into the calcium soap. Hence the loss of phosphorus and increase in calcium could be explained.

Preparation of the lead salt of a diglyceridephosphoric acid.

As a means of preventing the possible contamination of the regenerated product, it was decided to make the lead salt after the crude free acid had been first obtained. 2·6 g. of the crude calcium salt were converted into the corresponding free acid, and the latter in ether solution was shaken several times with an aqueous solution of lead acetate containing a little acetic acid. If some substance had been previously hydrolysed to yield fatty acids these would not be capable of decomposing lead acetate. Hence they would remain as free acids along with the lead salt of the diglyceridephosphoric acid in the ether, and when the salt was precipitated by the addition of four volumes of alcohol, they would remain in the ether-alcohol mother-liquor (see Appendix 2). (It was found that the ether-alcohol mother-liquor retained the deep red colour which had been characteristic of the solution of the crude and regenerated calcium salts mentioned above.) The precipitated lead salt was greyish and dissolved in ether to give a pale yellow-brown solution. On precipitating a second time the ether-alcohol mother-liquor was water-clear.

The yield of the lead salt after two precipitations from the ether-alcohol was 1·707 g.

Lead salt of diglyceridephosphoric acid H:

0·0755 g. substance : 0·1414 g. CO_2, 0·0493 g. H_2O, 0·0240 g. ash.
0·1707 g. ,, 0·0550 g. $PbSO_4$.
0·1707 g. ,, 0·0207 g. $Mg_2P_2O_7$.

	C	H	Pb	P	Ash
Found	51·07	7·38	22·02	3·43	31·60
Theory	51·47	8·14	22·80	3·41	32·33 (calc. as $Pb_2P_2O_7$)

This lead salt was contaminated with 0·65 % of Fe determined colorimetrically by comparison with standard $Fe_2(SO_4)_3$ treated with $K_4Fe(CN)_6$: it also contained 0·17 % of N determined by the micro-Kjeldahl method. The significance of the presence of these elements will be discussed later (see Appendix 1).

These analytical results were considered satisfactory for a compound of such a type as this. This preliminary work established a method of purifying the crude calcium salt and the next batch of material was accordingly treated in the same way.

ANALYSIS AND CONSTITUTION OF THE FREE DIGLYCERIDEPHOSPHORIC ACID.

Preparation of the free diglyceridephosphoric acid.

Batch M. From 64 g. of original ether-soluble material, 7·80 g. of crude calcium salt were obtained by the method already described. This sample had Ca 6·13 %, P 4·78 %; Ca : P = 1 : 1. The whole of this salt in ether solution was shaken out with dilute sulphuric acid to convert it into the corresponding acid, which in turn was shaken with lead acetate containing a little free acetic acid. The washed ether solution was concentrated to 250 cc. and two volumes of absolute alcohol added. The lead salt was filtered off, redissolved in 70 cc. of anhydrous ether and reprecipitated by the addition of three volumes of absolute alcohol. The yield of purified lead salt was 5·00 g.

Lead salt of diglyceridephosphoric acid M:

0·0769 g. substance : 0·1432 g. CO_2, 0·0526 g. H_2O, 0·0234 g. ash.

	C	H	Ash
Found	50·06	7·49	30·00
Theory	51·47	8·14	32·30

4·80 g. of this salt yielded 3·51 g. of free acid.

Diglyceridephosphoric acid M:

0·0832 g. substance : 0·1997 g. CO_2, 0·0742 g. H_2O.
0·2140 g. ,, 0·0334 g. $Mg_2P_2O_7$.
0·0507 g. ,, required (micro-Kjeldahl) 0·85 cc. 0·01 *N* acid.
(Fe 0·59 %)

	C	H	P
Found	65·48	9·91	4·34
Theory	66·57	10·94	4·40

Analysis of the diglyceridephosphoric acid. 3·06 g. of the acid dissolved in 40 cc. of mixed alcohol and ether were emulsified with 20 cc. of water and slowly run drop by drop into 200 cc. of boiling water containing 6 g. of barium hydroxide. The boiling was continued for 2 hours and on cooling the barium soaps were removed by filtration. The barium soaps were ground twice with water and filtered. The combined filtrate and washings were treated with CO_2 to remove excess barium, the barium carbonate filtered off and washed with water. The combined filtrate was boiled to precipitate any bicarbonate, cooled and filtered. The final filtrate was reduced to a volume of 25 cc. *in vacuo*, and $2\frac{1}{2}$ volumes of absolute alcohol added. The precipitate, which appeared as a curd, was washed with alcohol and ether and dried *in vacuo*. The yield of crude barium glycerophosphate was 0·73 g. (55 % of theory). A control experiment in which 1 g. of barium glycerophosphate, prepared from commercial glycerophosphoric acid, was used, gave a similar percentage yield when submitted to exactly similar treatment.

Analysis of the barium glycerophosphate. 0·73 g. of the salt dissolved readily in about 10 cc. of water, giving a slightly yellow and faintly opalescent solution. It was filtered and two volumes of absolute alcohol were added to the filtrate. The precipitate was curdy as before and after washing was dried at 107°. Yield 0·63 g.

0·0860 g. substance : 0·0368 g. CO_2, 0·0201 g. H_2O, 0·0624 g. ash.
0·1582 g. substance on ignition gave 0·1122 g. ash, equivalent to 0·0155 g. P or 9·81 %: converted to the sulphate it gave 0·1154 g. $BaSO_4$, equivalent to 0·0679 g. Ba or 42·86 %. This amount of Ba is equivalent to 0·1110 g. $Ba_2P_2O_7$.

	C	H	Ba	P
Found	11·66	2·59	42·86	9·81
$C_3H_7O_6PBa\frac{1}{2}H_2O$	11·40	2·53	43·42	9·81

It was necessary to boil the aqueous solution twice with charcoal before it became clear enough for the optical rotation to be determined.

$$[\alpha]_D^{18} = +\ 1{\cdot}0° \ (l = 2,\ c = 3).$$

Fränkel and Dimitz [1909] isolated a dextrorotatory barium glycerophosphate from brain cephalin and Trier [1913] a similar product from seed lecithin. In each case the salt was purified by repeated solution in water and precipitation with alcohol. Levene and Rolf [1919, 1926] have since shown that in both these cases the dextrorotation was due to an impurity which cannot be removed by this treatment. They purified the barium salt by conversion into the lead salt, decomposed the lead salt by means of H_2S and reconverted the glycerophosphoric acid into the lead salt. This operation was repeated until a laevorotatory solution was obtained. From this solution a barium salt was precipitated by means of alcohol; this salt had the normal laevorotation.

Owing to the small amount of barium salt which we obtained it was not possible to purify it by this method, so that the question of its rotation must

be left open until such time as we are able to obtain sufficient quantities of material for this purpose. From Levene and Rolf's work it is to be inferred that the barium salt, after the lead treatment, will be laevorotatory.

The original mother-liquor from which the barium glycerophosphate had been precipitated by alcohol was evaporated to dryness *in vacuo*. The residue weighed 0·073 g., the major part of which was barium glycerophosphate. Exhaustive extraction with absolute alcohol dissolved 0·021 g. If the small amount of nitrogen present in the free acid was due to lecithin then we should expect to find evidence of choline in this alcoholic extract. Actually it was found to give no precipitate with either alcoholic $HgCl_2$ or H_2PtCl_6. All the fractions of the barium glycerophosphate mother-liquor were then united and the total nitrogen determined by Kjeldahl. 0·3 cc. of 0·1 *N* acid were required, equal to 0·00042 g. of nitrogen and equivalent to 0·014 % of the total weight of free acid taken for hydrolysis. The significance of this result is discussed in Appendix 1.

The fatty acids of the diglyceridephosphoric acid. The barium soaps obtained during the baryta hydrolysis just described were decomposed with HCl under ether, the ether extract was thoroughly washed with water and evaporated to dryness. Yield 2·30 g. or 74·9 % of the diglyceridephosphoric acid taken (theory for C_{18} saturated acids 2·48 g. or 80·7 %).

0·1654 g. dissolved in abs. alcohol were neutralised by 0·81 cc. of 5/7 *N* HCl. Neutralisation value 196.
0·0920 g. absorbed 0·1254 g. iodine (Wijs). Iodine value 136.

These figures for the neutralisation and iodine values are in good agreement with those obtained in earlier (unrecorded) experiments. The amount of the fatty acids at our disposal was far too small for any reliable data to be obtained as to their nature. It seemed worth while however to attempt to determine whether they are in any way parallel to those of lecithin, which Levene has shown, in a large number of papers dealing with the acids of lecithins obtained from various sources, to consist of equal molecular proportions of unsaturated and saturated acids. Further, the work of that author and his colleagues on lyso-lecithins seems to exclude definitely the occurrence either of two saturated or two unsaturated acids in any one molecule of lecithin. The conclusion which Levene draws is that in each lecithin there is one saturated and one unsaturated acid.

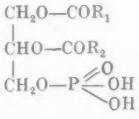
Accordingly 1·8 g. of the fatty acids was converted into the lead soaps, which were extracted with ether. There remained insoluble in ether only a very small amount of material, which yielded on decomposition with acid 0·1 g. of saturated acid, which was hard and white. It would appear therefore that the saturated and unsaturated acids are not present in equivalent proportions, but that the unsaturated acids are present in far greater amount. This result, which will necessarily need confirmation on larger quantities of material, tends to show that this compound differs from lecithin in that in any one molecule there may be two unsaturated acids present.

The fatty acids obtained by decomposing the ether-soluble lead soaps were brominated in anhydrous ether solution. On standing overnight on ice 0·1 g. of solid material separated. This, after careful removal of the excess bromine, had a melting point of 179°. 0·0690 g. was found by Stepanoff's method to contain 0·0432 g. of bromine equivalent to 62·6 %. These results indicate the presence of a C_{18} acid with three double bonds. A consideration of the iodine value of the original mixed acids, together with the amounts of saturated acid and acid with three double bonds obtained, shows that the majority of the remainder must consist of linolic acid and the residue of oleic acid. This is borne out by the fact that the residual brominated acids, which failed to crystallise, contain 47·7 % of bromine.

0·1347 g. substance absorbed 0·0644 g. Br.

	Br
Found	47·7
Theory for C_{18} acids with (A) one double bond	35·9
,, ,, (B) two double bonds	53·3

Structure of the diglyceridephosphoric acid. Sufficient evidence has been adduced to enable a structural formula to be ascribed to the diglyceridephosphoric acid. From it there has been isolated an optically active barium glycerophosphate and hence the phosphoric acid is linked to the glycerol in the α-position. The yield of fatty acids has proved that there are two molecules of fatty acid to one of glycerophosphoric acid, and it would seem reasonable to suggest that the structural formula for this substance is similar to that accepted for lecithin, and is

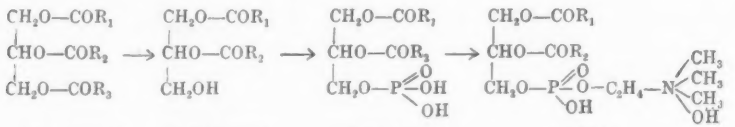


A substance of this type, namely $\alpha\beta$-distearylglycerophosphoric acid, was synthesised by Hundeshagen [1883] and such compounds have been frequently used since, as intermediate products in attempts to synthesise lecithin itself. So far as we are aware their occurrence has not been reported in natural products.

It is of interest to note however that a preparation of a phosphatide-like substance was obtained from the leaf by Winterstein and Stegmann [1909]. These workers extracted 4 kg. of air-dried leaves of *Ricinus* with ether for two or three days at 30°. The essential point of their subsequent procedure was to evaporate the ether extract to dryness and extract with hot alcohol. The insoluble residue, after solution in ether, was precipitated with alcohol. The substance, after drying in a vacuum desiccator, contained 5·27 % of P and 6·74 % of CaO. Beyond showing that it did not contain any sugar these workers went no further. This substance would appear to be a crude calcium

salt similar to that described in this paper. Also Stern and Thierfelder [1907] reported that a phosphatide fraction from egg-yolk contained 3·96 % P, 1·37 % N and 1·03 % Ca. They were convinced that the calcium was present in organic combination.

Although the natural occurrence of a diglyceridephosphoric acid had not been reported, it has been suggested by Trier [1912] that the successive stages in the synthesis of plant lecithins are as follows:



Triglyceride — $\alpha\beta$-Diglyceride — $\alpha\beta$-Diglyceride-phosphoric acid — Lecithin

If the work reported in this paper be substantiated it may prove to be a link in the above scheme.

One further point needs discussion. It will be remembered that in the preparation of the original coagulum, the expressed juice is heated to 70° to coagulate the cytoplasm, and the possibility of this process having caused the partial hydrolysis, and the substitution of the choline present in lecithin by calcium, remains to be considered. The p_H of the expressed juice was about 6, and it seemed improbable that heating a suspension of lecithin to 70° for a few minutes in a solution of such low effective acidity would cause such a reaction to occur, and to proceed to completion.

To prove this point a batch of 7 kg. of leaves was minced in the usual way and the green solution containing the colloidal cytoplasm filtered instead of being warmed to 70° to coagulate the cytoplasm. Part of the protein and all the ether-soluble material was retained in the upper region of the filter pad. This green slime was scraped off and pressed in the Buchner press to a hard cake. This material, which had thus been prepared without the application of heat, was directly extracted with ether; the ether extract weighed 14·5 g. and yielded 2·6 g. of crude calcium salt.

0·0972 g. substance : 0·2090 g. CO_2, 0·0759 g. H_2O, 0·0182 g. ash.
0·2596 g. ,, 0·0503 g. $CaSO_4$, 0·0032 g. Fe_2O_3.
0·2596 g. ,, 0·0414 g. $Mg_2P_2O_7$.

	C	H	Ca	P	Ash	(Fe)
Found	58·63	8·67	5·70	4·65	18·73	0·86

This result would make it appear certain that the calcium salt of the diglyceridephosphoric acid pre-existed in the leaf cell, and that it was not a product of phospholipin decomposition.

Properties of the diglyceridephosphoric acid, and the preparation and properties of some of its salts.

Free acid. Prepared by evaporation of the ether solution at a temperature not exceeding 30°, it is a pale brown sticky mass, which does not readily

absorb moisture if exposed to the air, but rapidly darkens due to oxidation. Thus prepared it is readily soluble in the usual organic solvents, especially ether. An alcohol or acetone solution can be considerably diluted with water without the formation of an emulsion. Slow decomposition occurs if the free acid or any of its salts are dried *in vacuo* at 100°, giving rise to products that are no longer soluble in ether.

Lead salt. Two analyses of this salt have already been quoted. A third preparation made from Batch G by the methods described above gave the following analysis:

0·0946 g. substance : 0·1735 g. CO_2, 0·0608 g. H_2O, 0·0293 g. ash.
0·1642 g. „ 0·0524 g. $PbSO_4$ and 0·0204 g. $Mg_2P_2O_7$.

	C	H	Pb	P	Ash
Found (Prep. G)	50·01	7·14	21·80	3·46	30·86
„ („ H)	51·07	7·38	22·02	3·43	31·60
„ („ M)	50·06	7·49	—	—	30·00
Theory	51·47	8·14	22·80	3·41	32·38

When precipitated by absolute alcohol from a solution in anhydrous ether and dried *in vacuo* at 30° it is obtained as brown flakes resembling gelatin, which become sticky under pressure. It is readily soluble in ether, from which it is almost completely precipitated by two volumes of alcohol or less completely by four volumes of acetone. The dry product darkens rapidly when exposed to the air.

Barium salt. Prepared from an ether solution of lead salt H by converting into the free acid and shaking with barium acetate.

0·2456 g. substance : 0·0322 g. $Mg_2P_2O_7$.
0·1228 g. „ 0·0352 g. $BaSO_4$.

	Ba	P
Found	16·80	3·65
Theory	16·37	3·69

Calcium salt. Prepared from an ether solution of lead salt G by converting into the free acid and shaking with calcium acetate.

0·0488 g. substance : 0·1154 g. CO_2, 0·0397 g. H_2O, 0·0089 g. ash.

	C	H	Ash
Found	64·47	9·04	18·03
Theory	63·10	10·10	17·17

The barium and calcium salts have properties similar to those of the lead salt.

Appendix 1.

On the possible presence of lecithin.

It will have been noticed that in all the preparations there has been present a small amount of nitrogen (0·15 to 0·3 %). This nitrogen, which persists and is not readily removed by the many purification treatments, presumably cannot be present in the molecule itself, as this would entail a molecular weight

of 7 or 8 thousand. The presence of nitrogen in the phospholipin fraction is of course to be expected, and we thought naturally that this nitrogen represented lecithin. Throughout the work, however, we have failed to obtain any evidence of the presence of that substance. On no occasion has a precipitate been obtained when treating an alcoholic solution of the free acid with alcoholic $CdCl_2$. (This test cannot be carried out on the salts which are insoluble in alcohol.) Further this free acid, containing nitrogen sufficient to account for, say, 10 % of lecithin, is very soluble in cold acetone. Another impurity has also persisted. The small quantity of iron present in the crude calcium salt is not removed when the latter is converted into the free acid. Again, after the hydrolysis with baryta in the preparation of the barium glycerophosphate the barium soaps formed a precipitate as they are insoluble in water. The fatty acids which were prepared by acidification of these soaps under ether contained practically all the nitrogen and iron present in the original substance. Hence, as most of the nitrogen was not water-soluble after hydrolysis, it cannot be present as choline, and this is again evidence of the absence of lecithin. Another interesting point arises here, namely, that the iron and nitrogen have appeared together in the same fraction of the hydrolysis products and this may indicate the presence of ether-soluble iron- and nitrogen-containing substances which had been unaffected by the hydrolysis, and which, being water-insoluble, have precipitated along with the barium soaps. The evidence thus seems to negative the possibility of lecithin being present in this ether extract of leaf cytoplasm—a very interesting result.

Appendix 2.

The possible presence of the calcium salt of a monoglyceridephosphoric acid.

The point which will now be discussed is the significance of the fact that when the crude calcium salt in ether solution is shaken with acid, part of the phosphorus originally present passes into the aqueous solution. This fraction was usually 20–40 %. As to the nature of the substance from which it is derived, we have no positive evidence, but a number of isolated observations seem to throw some light on the question. Firstly, if the regenerated calcium salt is again shaken out with acid there is no further loss of phosphorus, which would suggest that the phosphorus which passes into the aqueous solution is derived from a second phosphorus-containing body and not from the calcium salt of the diglyceridephosphoric acid. Secondly, the regenerated calcium salt obtained by decomposing the crude salt with acid, and shaking the free acid so obtained with lime water always gives a high value for calcium and a low value for phosphorus. We may surmise from this that whatever has been hydrolysed by the shaking with acid and thereby has lost its phosphoric acid, must have given rise at the same time to other products of a fatty nature, which are able to unite with the lime. We suspected that these acidic sub-

stances might be fatty acids, and this was confirmed to some degree as follows. When the free acid in ether solution is shaken with aqueous lead acetate the lead salt of the diglyceridephosphoric acid is formed, and can be precipitated by the addition of excess of alcohol to the ether solution. If it be filtered off, the mother-liquors on evaporation leave a residue consisting of small amounts of lead salt together with another substance. This latter can be removed by solution in cold alcohol, and can be titrated in that solvent with NaOH. From the neutralised product there was obtained from batch M, discussed above, 1·22 g. of crude fatty acid having a neutralisation value of 177. Hence the second substance, which breaks down on treatment with acid, contains calcium and phosphorus, and gives rise to fatty acids. Further, since the calcium and phosphorus in the original crude calcium salt are both above the theoretical for the calcium salt of a diglyceridephosphoric acid the other substance present must contain a high percentage of calcium and phosphorus. Since this latter gives rise to fatty acids on hydrolysis, it seems possible that the second substance may be the calcium salt of a monoglyceridephosphoric acid, which would contain Ca 8·42 %, P 6·53 %. The presence of this substance in the crude Ca salt would raise both the calcium and phosphorus, and it seems reasonable to suggest that if such a substance in ether solution were treated with aqueous acid, it would tend to undergo hydrolysis much more easily than would the compound containing two fatty acid radicles in the molecule. No figures have been quoted for the amount of phosphorus passing into the aqueous solution, for, when the calcium salt has been shaken with mineral acid and the ether solution of the free acid is being shaken with water to remove the last traces of mineral acid, emulsions always occur. These emulsions are difficult to break, and it has been our practice to do this by adding small quantities of alcohol. There is little doubt that some of the loss of phosphorus is due to the removal of small quantities of free acid owing to its solubility in water containing alcohol. This fact has made it seem useless to quote figures for the phosphorus of the aqueous extract.

We hope to investigate this calcium salt of a monoglyceridephosphoric acid in greater detail at a later date. It is possible that this supposed salt did not pre-exist in the leaf cell, but was formed from the calcium salt of the diglyceridephosphoric acid by the action of enzymes or heat.

Summary.

The so-called phospholipin fraction—obtained by adding acetone to an ether solution of a fat—contains no phospholipins in the case of the ether-soluble substances of the cytoplasm of the cabbage leaf. All the phosphorus is present in combination with calcium, glycerol and fatty acids. Nitrogen is virtually absent.

The main constituent of the fraction is the calcium salt of a diglyceride-phosphoric acid. The preparation and analysis of this acid, and the preparation and properties of some of its salts are given.

REFERENCES.

Fränkel and Dimitz (1909). *Biochem. Z.* **21**, 337.
Hundeshagen (1883). *J. prakt. Chem.* (2) **28**, 219.
Levene and Rolf (1919). *J. Biol. Chem.* **40**, 1.
—— —— (1926). *J. Biol. Chem.* **68**, 285.
Stern and Thierfelder (1907). *Z. physiol. Chem.* **53**, 360.
Trier (1912). Über einfache Pflanzenbasen und ihre Beziehungen zum Aufbau der Eiweissstoffe und Lecithine (Berlin).
—— (1913). *Z. physiol. Chem.* **86**.
Winterstein and Stegmann (1909). *Z. physiol. Chem.* **58**, 527.

XXXIV. ANALYSIS OF PROTEINS. VIII.
ESTIMATION OF CYSTINE IN THE MODIFIED VAN SLYKE METHOD.

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In the shortened form of the Van Slyke method of analysis of proteins adopted by Plimmer and Rosedale [1925], in which the determination of the various amino-acids was carried out without removal of phosphotungstic acid from the solutions, the estimation of cystine was not attempted. The direct determination of cystine has now been investigated with satisfactory results.

The estimation of cystine in proteins by this method depends also upon its behaviour in the various stages of the process. Van Slyke [1911] found that only 50 % of the cystine, after hydrolysis with 20 % hydrochloric acid for 24 hours, was precipitated by phosphotungstic acid. It was shown by Hoffman and Gortner [1922] that cystine on boiling with hydrochloric acid was slowly changed into an "isomeric" form which was more soluble, was optically inactive, did not crystallise in the characteristic form of ordinary cystine, and formed a much more soluble phosphotungstate. These observations have been confirmed and extended. The sulphur content of the diamino-fraction really represents from 40 to 50 % of the cystine in the protein. Either a correction could be applied assuming that half the cystine was precipitated by phosphotungstic acid, or a determination of the sulphur content of the monoamino-fraction could be made for the amount of the non-precipitated "isomeric" cystine. This latter determination involves the assumption that no other sulphur-containing compound is present. In view of the isolation of another sulphur-containing amino-acid by Mueller [1923] the sulphur content of the monoamino-fraction cannot be regarded as being due only to "isomeric" cystine. It has been possible for us to estimate the sulphur content of the solutions of the several egg proteins analysed by Plimmer and Rosedale [1925]. The amounts of sulphur in the monoamino-fractions seem to bear no relation to the amounts in the diamino-fractions, but tend to indicate the presence of another sulphur compound which is not precipitated by phosphotungstic acid.

Though the sulphur estimation of the diamino-fraction does not represent the actual amount of cystine in a protein, its estimation is necessary in order to arrive at the amount of lysine. The presence of cystine in this fraction gives

erroneous values for arginine, as it gives off, according to Van Slyke [1911], 18 % of its nitrogen on boiling with 40 % caustic potash. The decomposition has been found to be rather less on boiling with 20 % caustic soda, under the conditions laid down for arginine by Plimmer [1916]. It is greater in the presence of phosphotungstic acid.

Plimmer and Rosedale [1925] found that the monoamino-fraction gave off ammonia on boiling with caustic soda and attributed its origin to arginine which had not been precipitated by phosphotungstic acid. The amount of sulphur in these fractions is too small to account for the quantity of ammonia. On standing for over 15 months these solutions have deposited small quantities of crystals. Their examination has shown them to consist of a mixture of diamino-acids and the solution has still given off ammonia on boiling with caustic soda. The monoamino-acids, glycine, alanine, tyrosine and proline have been boiled with caustic soda and do not evolve any ammonia. It has not yet been possible to test a hydroxyamino-acid, so that the origin of the ammonia still appears to be from non-precipitated arginine. It is most likely that part of the arginine of the protein is racemised on hydrolysis and that racemic arginine forms a more soluble phosphotungstate than the active arginine.

Experimental.

The cystine for these experiments was prepared from feathers. The feathers were hydrolysed by boiling with twice their weight of conc. hydrochloric acid for 6 hours. The acid solution was evaporated *in vacuo* to remove hydrochloric acid as far as possible. The residue was dissolved in water, filtered from black material, boiled with charcoal, again filtered, nearly neutralised with sodium hydroxide (Hoffman and Gortner) and treated with sodium acetate until neutral to Congo red (Folin). The brown product was purified by boiling in 5 % (vol.) hydrochloric acid with charcoal, filtering and precipitating with sodium acetate. The colourless product was then treated with alcohol saturated with hydrogen chloride to remove tyrosine [Plimmer, 1913] and crystallised several times from dilute hydrochloric acid by addition of sodium acetate. After drying in a desiccator, the cystine gave the following figures on analysis:

Found (mean of 5 analyses): N = 11·5 %, S = 26·3 %.
Calculated for $C_6H_{12}O_4N_2S_2$: N = 11·67 %, S = 26·67 %.

Both analytical figures thus indicated a purity of 98·6 %. The sample was found to leave no ash on incineration, and microscopical examination showed only the typical hexagonal crystals of cystine. It is not possible at present to explain the low analytical data for the purified cystine. It may be noted that Hoffman and Gortner obtained similar analytical figures for pure cystine indicating a purity of 97·5 %[1].

[1] [Note added February 7, 1927.] Dr W. H. Hurtley has suggested to us that cystine pre-

I. *Estimation of cystine sulphur in presence of phosphotungstic acid.*

In the Van Slyke method of protein analysis 15 g. of phosphotungstic acid are used in precipitating the diamino-fraction. Known weights of cystine have been oxidised in presence of this large quantity of phosphotungstic acid by the Benedict-Denis method.

On treating the oxidised residue with dilute hydrochloric acid, it was found that the tungstic acid, produced during the incineration, gave a very fine white precipitate which settled out only slowly and was very difficult to separate by filtration. A further quantity of tungstic acid also came down on adding barium chloride for precipitation of the barium sulphate. This treatment was thus not a practical one for the purpose.

As tungstic acid forms a very insoluble yellow oxide on precipitation from strongly acid solutions, whereas it gives the white hydrated oxide in dilute solution, the next experiments were made using conc. hydrochloric acid for solution of the oxidised residue. The greater part of the tungstic acid was then obtained as yellow oxide which could be easily filtered off on a hardened paper and washed with conc. hydrochloric acid. On diluting the solution with water, a small quantity of the white oxide was precipitated; on warming it settled out and could be filtered off and washed with dilute hydrochloric acid. A clear solution was thus obtained for precipitation with barium chloride. The last traces of tungstic acid are not however always removed by this treatment and come down with the barium sulphate. These small amounts are casily soluble in dilute alkali and can be removed by washing the precipitate of barium sulphate with dilute ammonia.

As a routine, the procedure may be described as follows. The solution of cystine phosphotungstate (20 cc. diamino-fraction, or 50 cc. monoamino-fraction) is evaporated to dryness with 5 to 10 cc. of the Benedict-Denis reagent, preferably on a hot plate, until distinct charring is seen. The residue is then heated over a flame for 15 minutes. The oxidised material is boiled with 25 cc. of conc. HCl. After standing (most conveniently overnight) the solution is filtered from insoluble yellow tungstic oxide through a 9 cm.

cipitated from acid solution with sodium acetate may retain traces of acetic acid and thus give low analytical figures for nitrogen and sulphur.

A sample of cystine prepared by Dr Hurtley by precipitation with sodium hydroxide and kindly supplied to us gave the following data:

0·1 g. gave 11·55
0·1 g. gave 11·75 } 11·65 cc. $N/14$ NH_3 $\quad N = 11{\cdot}65\ \%$

0·1 g. gave 0·1941 g. $BaSO_4$ $\quad S = 26{\cdot}65\ \%$.

On recrystallising our own specimen by precipitation from acid with sodium hydroxide, it gave the correct figures:

0·1 g. gave 11·65
0·1 g. gave 11·65 } 11·65 cc. $N/14$ NH_3 $\quad N = 11{\cdot}65\ \%$

0·1 g. gave 0·1930
0·1 g. gave 0·1943 } 0·1936 g. $BaSO_4$ $\quad S = 26{\cdot}60\ \%$.

It thus appears that the low values for cystine nitrogen and sulphur are due to precipitation with sodium acetate and retention of acetic acid.

hardened paper and the precipitate washed with from 25 to 50 cc. of hot conc. HCl and then with hot 50 % HCl. The solution is diluted with 200 to 500 cc. of water. The white precipitate which forms settles out as yellow oxide on warming and is filtered off and washed with dilute HCl (1:1). The clear solution is precipitated with barium chloride. The barium sulphate is washed thoroughly with water and then with dilute ammonia to remove the last traces of tungstic acid. The final treatment of the barium sulphate is carried out by the Folin method of washing with alcohol, burning the paper in a crucible and heating to redness. The following results have been obtained:

0·25 g. cystine + 15 g. phosphotungstic acid gave 0·4806 g. $BaSO_4$
0·25 g. „ „ „ „ 0·4794 g. „ } S = 26·36 %.

These figures correspond with those for the cystine used above, S = 26·31 %.

II. *Estimation of cystine sulphur in cystine phosphotungstate.*

As the main object of these experiments was the estimation of cystine in the phosphotungstic acid precipitate obtained in the modified Van Slyke procedure, known quantities of cystine (0·25 g.) in hydrochloric acid solution were precipitated by an excess of phosphotungstic acid in a total volume of 200 cc. Under these conditions the precipitate came down slowly. After standing for 5 days, the precipitate was filtered off on a special glass filter and washed with 5 portions of 10 cc. dilute hydrochloric acid. The precipitate was dissolved in sufficient caustic soda solution and the volume made up to 100 cc. 20 cc. portions of the solution were taken for total nitrogen and for sulphur determinations.

		Mean			Mean
N	{5·5 5·65 / 5·65 5·6}	5·6 cc. $N/14$ NH_3,	S	{0·0930 0·0934 / 0·0928 0·0928}	0·0930 g. $BaSO_4$.

For the corresponding quantity of 0·05 g. cystine the quantities should be 5·75 cc. $N/14$ NH_3 and 0·0958 g. $BaSO_4$.

Both the nitrogen and the sulphur estimations showed that 97·4 and 97·1 % of the cystine were precipitated. The figures correspond with those of Hoffman and Gortner, who determined the extent of the precipitation by nitrogen determinations.

The filtrate from the precipitate of the cystine phosphotungstate was also examined for nitrogen and sulphur: 50 cc. of the filtrate made up to 250 cc. gave:

0·1 and 0·2 / 0·15 „ 0·1 } cc. $N/14$ NH_3 0·0022 and 0·0008 g. $BaSO_4$.

The nitrogen was thus equal to 2·5 % of the total nitrogen. The amounts of barium sulphate were insufficient for accurate weighing.

III. *Behaviour of cystine during hydrolysis.*

The observations of Van Slyke [1911] and of Hoffman and Gortner [1922] upon the action of hydrochloric acid upon cystine have been repeated and extended. One portion of 1 g. cystine and one portion of 2·5 g. cystine were boiled for 36 hours under a reflux with 100 and 250 cc. of 25 % HCl. The

solutions became brown in colour, deposited a dirty brown precipitate and gave a distinct odour of hydrogen sulphide. The excess of hydrochloric acid was distilled off *in vacuo* and the volumes made up to 100 cc. and 250 cc. A complete series of analyses was then carried out by the Plimmer-Rosedale procedure, using quantities of the solutions containing 0·25 g. cystine. The following are the data:

		I		II	In % of	
					Total N	Total S
Hydrolysed solution	Total N	5·6		5·6 cc. *N*/14	*Mean*	
		5·7		5·6 cc. „		
	Total S	0·4700		0·4711 g. $BaSO_4$	97·4	98·0
		0·4692		0·4622 g. „		
Amide N		1·3	1·2	1·25 cc. *N*/14	4·3	—
Humin N		0·9	0·65	0·85 cc. „	2·7	—
Phosphotungstic precipitate:						
20 cc. out of 100 cc.		2·1	2·2	2·0 cc. „	—	—
		0·040	0·0412	0·0386 g. $BaSO_4$	36·5	41·8
Phosphotungstic filtrate:						
50 cc. out of 250 cc.		3·2	3·25	2·85 cc. *N*/14	—	—
		0·0538	0·0542	0·0528 g. $BaSO_4$	53·9	56·2

Cystine is thus decomposed by boiling with acid. About 7 % of its nitrogen was lost as amide and humin nitrogen. The sulphur content of the diamino-fraction represents about 40 % of the cystine sulphur; 56 % of the sulphur is found in the monoamino-fraction.

IV. *Estimations of the sulphur content of egg proteins.*

The remainders of the diamino- and monoamino-fractions of the egg proteins analysed by Plimmer and Rosedale [1925] have been analysed for their sulphur content by the method described above. The figures for barium sulphate have been changed to figures for cystine nitrogen by using the equivalent of 1 mg. $BaSO_4$ = 0·06 mg. cystine N. The data are:

	Diamino-fraction 100 cc. g.	Monoamino-fraction 250 cc. g.
Egg-yolk	0·0004	0·0170
Egg-white	0·0038	0·0124
Ovomucoid	0·0026	0·0183
Egg-membrane	0·0065	0·0133
Casein	0·0000	0·0010
Gelatin	0·0002	0·0075

It is of interest to notice the low amount of cystine nitrogen in the diamino-fraction of egg-yolk. The two phosphoproteins, caseinogen and vitellin, thus resemble each other also in their cystine sulphur content.

From the results of the analyses on boiling cystine with hydrochloric acid it might have been expected that the sulphur content of the monoamino-fraction would have been $\frac{3}{2}$ times that of the diamino-fraction, but except in the case of the egg-membrane the amount is considerably greater. It may therefore be inferred that another sulphur compound is present in all the proteins. Egg-membrane appears to contain mostly cystine sulphur.

V. *The presence of arginine in the monoamino-fraction of the egg proteins.*

The monoamino-fractions of the egg proteins and caseinogen analysed by Plimmer and Rosedale [1925] have, on standing for over 15 months, deposited small quantities of crystals. These crystals have been filtered off, dissolved in dilute soda, and analysed for total nitrogen and arginine nitrogen and in one case cystine sulphur. The filtrates have also been analysed for total and arginine nitrogen. The figures are:

	Volume of remaining solution cc.	Original		Filtrate		Precipitate		
		T.N.	Arg. N.	T.N.	Arg. N.	T.N.	Arg. N.	Cyst.
Caseinogen	1875	0·2086	0·0052	0·2114	0·0056	0·0287	0·0098	—
Egg-yolk	600	0·1666	0·0126	0·0166	0·0098	0·0042	0·0036	—
Egg-white	610	0·2016	0·0126	0·1974	0·0098	0·0042	0·0036	—
Ovomucoid	740	0·1946	0·0238	0·1862	0·0238	0·0140	0·0028	—
Egg-membrane	710	0·1960	0·0168	0·1890	0·0154	0·0182	0·0049	0·0020

There has been a small diminution, as expected, in the total nitrogen of the solutions and little change in the amount of arginine nitrogen. The precipitate consists only in part of arginine phosphotungstate. The amounts of "isomeric" cystine in these solutions (section IV) are not sufficient to account for the whole of this arginine nitrogen.

The possibility that the ammonia arose from monoamino-acids, though they were shown to be stable to caustic potash by Van Slyke, has again been tested by boiling solutions of glycine, alanine, proline, and tyrosine with 20 % caustic soda. No ammonia was evolved in any of these experiments. Specimens of hydroxyamino-acids have not been available for testing with caustic soda. The original conclusion that the ammonia arose from non-precipitated arginine is thus strengthened. It is possible that the arginine in the solution comes from racemic arginine which may form a more soluble phosphotungstate.

VI. *Action of sodium hydroxide on cystine.*

The action of caustic potash upon cystine was tested by Van Slyke [1911], who found that 18 % of its nitrogen was given off. To complete the series of observations upon the behaviour of cystine in the Van Slyke method, the action of 20 % caustic soda for 6 hours, the conditions found by Plimmer [1916] for the decomposition of arginine, has been tried upon cystine, "hydrolysed" cystine, cystine precipitated by phosphotungstic acid and unprecipitated "isomeric" cystine. The results were as follows:

Solution boiled with 20 % caustic soda	Total N of solution cc. *N*/14	Ammonia evolved cc. *N*/14		Mean	% of total N
10 cc. 0·1 % pure cystine	11·5	1·0	1·2	1·1	9·6
10 cc. 0·1 % hydrolysed cystine	11·2	1·5	1·3	1·3	11·8
20 cc. cystine phosphotungstate in soda	2·1	0·5	0·4	0·45	21·4
50 cc. filtrate from phosphotungstate	3·1	0·4	0·45	0·4	13·7

The decomposition of pure cystine is thus less with 20 % soda than found by Van Slyke with 40 % potash. The amount is a little greater after hydrolysis. In presence of phosphotungstic acid, the amount of decomposition is greater. This decomposition of cystine by boiling with soda will give results for arginine which are too high. A deduction of one-fifth of the cystine nitrogen should be made before calculating the arginine nitrogen.

Summary.

1. The sulphur of cystine can be estimated in the presence of phosphotungstic acid by a suitable alteration of the Benedict method of determining sulphur.

2. 97 % of pure cystine is precipitated by phosphotungstic acid.

3. Cystine is changed by boiling with acids, losing 7 % of its nitrogen; only 40 % is then precipitated by phosphotungstic acid.

4. Cystine, on boiling with caustic soda, loses 10 % of its nitrogen as ammonia. The decomposition reaches 20 % after boiling with acid and in presence of phosphotungstic acid.

5. The amounts of cystine sulphur in the egg proteins and caseinogen have been determined. The figures indicate the presence of another sulphur compound in these proteins.

6. The probable presence of arginine in the monoamino-fractions obtained in the Van Slyke method of analysis is emphasised by its precipitation as phosphotungstate when the solutions are kept. It is possibly racemic arginine.

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References.

Hoffman and Gortner (1922). *J. Amer. Chem. Soc.* **44**, 341.
Mueller (1923). *J. Biol. Chem.* **56**, 157.
Plimmer (1913). *Biochem. J.* **7**, 311.
—— (1916). *Biochem. J.* **10**, 115.
Plimmer and Rosedale (1925). *Biochem. J.* **19**, 1015.
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.

XXXV. CHANGES IN THE AMINO-ACIDS IN THE PROTEINS OF THE HEN'S EGG DURING DEVELOPMENT.

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Of the various changes which take place in the hen's egg during development into the chick those occurring in the protein have not yet been thoroughly investigated. The excellent review of all the work on "The Metabolism of the Developing Egg" by Needham [1925] does not refer to any investigation of the proteins. Several papers have been published upon changes in the ammonia and nitrogen content, and Needham [1926, 1] has published data upon the urea content of the developing egg, and he has also studied the uric acid and non-protein nitrogen content [1926, 2]. Fiske and Boyden [1926] have also worked upon the nitrogen metabolism of the chick embryo.

Sendju [1925] has determined the tyrosine and tryptophan content of the developing egg by colorimetric methods and the diamino-acid content by Kossel's method. A decrease was found in the tyrosine and tryptophan content, but no definite changes in the diamino-acid content.

With the improvements made in the Van Slyke method by Plimmer and Rosedale [1925] it was hoped it would be possible to detect changes in the amino-acid content which would indicate the origin of the ammonia, urea, uric acid and other simpler compounds.

Experimental.

Previous experiments upon the changes in the constituents of eggs during development had shown that no marked alteration was noticeable until about the 11th day of incubation. No attempt was therefore made to analyse the amino-acids at daily intervals. The fresh egg, the egg at the stage of 15 days' incubation and the hatched chick were used so as to secure information at the beginning, at the middle and at the end of the metamorphosis. The eggs in these experiments were from Light Sussex hens fed upon a diet of vitamealo, middlings and maize. Three eggs were used at each stage of the development. Sufficient material was thus obtained to allow of several analyses of the amino-acids by the Van Slyke method.

The contents of the fresh eggs, the eggs at 15 days, and the chicks after chloroforming and cutting up into small pieces were placed in 400 to 500 cc. of alcohol and treated in a similar way to that described by Plimmer and Scott [1909] in order to separate fats and lecithin and to coagulate the protein. The material after standing some days in alcohol was warmed and extracted first with alcohol and then with ether in a muslin bag suspended in a special extractor. The amounts of coagulated protein so obtained were:

From fresh eggs	17·0 g.
„ eggs at 15 days' incubation	20·6
„ hatched chicks	19·3

The alcohol and ether extracts were made up to 1000 cc. and the nitrogen in them determined by Kjeldahl's method. The amounts were:

From fresh eggs	0·1288 g.
„ eggs at 15 days' incubation	0·1344
„ hatched chicks	0·1190

These figures, if calculated per 100 g. of protein, show a decrease in the amount of alcohol- and ether-soluble nitrogen, but they probably have no relation to any changes in the proteins.

The coagulated protein at each stage was hydrolysed with 200 cc. of 25 % hydrochloric acid for 36 hours. The solution was evaporated *in vacuo* to remove hydrochloric acid as far as possible and the residue dissolved in water and made up to 520 cc.

The amino-acids were determined in 100 cc. portions of these solutions by the Van Slyke method as modified by Plimmer and Rosedale. Four analyses were made at each stage. The residual diamino- and monoamino-acid solutions were then combined and the analyses repeated. Sulphur estimations were also made by direct determination, as shown possible by Plimmer and Lowndes [1927].

The series of analyses has been repeated with one egg at each stage. These eggs were also from Light Sussex hens, but fed upon white rice, dried yeast, fish meal and cod-liver oil, from group XLV of birds in other experiments on nutrition.

The amounts of protein obtained were:

From a fresh egg	5 g.
„ an egg at 15 days' incubation	5
„ a hatched chick	7

After hydrolysis the solutions were made up to 250 cc. and two analyses of the amino-acids were made. The residues were combined for another estimation and a sulphur estimation.

Although it had been found by Plimmer and Phillips [1924] that the tyrosine content could not be determined by bromination on account of the presence of cystine, or some other compound which reacts with bromine, it was of some interest to ascertain the absorption of bromine by the monoamino-fraction at each stage of development. This estimation has been made for us by Mr L. R. Bishop.

The following are the data, all calculated in terms of nitrogen per 100 cc.:

Exp. 1. Total N. Stage: 0 0·4578 g.
„ 15 days 0·4928
„ hatched chick 0·4788

	Amide	Humin	Diamino N Total	Amino	Non-amino	Argi-nine	Histi-dine	Ly-sine	Monoamino-N Total	Amino	Non-amino	Argi-nine
Stage: 0												
(1)	0·0372	0·0073	0·1190	0·0708	0·0482	0·0651	−0·0009	0·0548	0·2954	0·3150	—	0·0224
(2)	0·0378	0·0067	0·1162	0·0690	0·0472	0·0644	−0·0016	0·0534	0·2926	0·2995	—	0·0210
(3)	0·0377	0·0067	0·1176	0·0642	0·0534	0·0616	0·0108	0·0452	0·2898	0·3050	—	0·0196
(4)	—	0·0063	0·1176	0·0693	0·0483	—	—	—	0·2954	0·3050	—	0·0210
Mean	0·0376	0·0067	0·1176	0·0683	0·0493	0·0637	0·0023	0·0516	0·2933	0·3061	—	0·0210
Residual solution	—	—	0·1190	0·0672	0·0518	0·0678	0·0014	0·0498	0·2926	0·3102*	—	0·0196
Stage: 15 days												
(1)	0·0435	0·0092	0·1302	0·0733	0·0569	0·0721	0·0042	0·0539	0·3010	0·3115	—	0·0210
(2)	0·0454	0·0087	0·1330	0·0737	0·0593	0·0707	0·0094	0·0529	0·2996	0·3150	—	0·0196
(3)	0·0423	0·0092	0·1316	0·0789	0·0527	0·0742	−0·0044	0·0618	0·3024	0·3242	—	0·0168
(4)	0·0399	0·0090	0·1302	0·0796	0·0506	0·0707	−0·0036	0·0631	0·2996	0·3160	—	0·0154
Mean	0·0428	0·0090	0·1312	0·0764	0·0548	0·0719	0·0013	0·0580	0·3006	0·3167	—	0·0182
Residual solution	—	—	0·1358	0·0721	0·0637	0·0756	0·0105	0·0497	0·3024	0·3212*	—	0·0168
Stage: hatched chick												
(1)	—	0·0077	—	—	—	—	—	—	0·2912	0·2780	0·0132	0·0154
(2)	0·0442	0·0080	0·1302	0·0679	0·0623	0·0742	0·0100	0·0460	0·2884	0·2833	0·0051	0·0154
(3)	0·0364	0·0078	0·1260	0·0753	0·0507	0·0833	−0·0177	0·0604	0·2884	0·2845	0·0039	0·0154
(4)	0·0381	0·0085	0·1316	0·0676	0·0640	0·0805	0·0004	0·0507	0·2912	0·3002	—	0·0168
Mean	0·0396	0·0080	0·1293	0·0703	0·0590	0·0793	−0·0006	0·0506	0·2898	0·2865	0·0033	0·0158
Residual solution	—	—	0·1330	0·0679	0·0651	0·0819	0·0055	0·0456	0·2870	0·2790*	0·0080	0·0154

* On repeating at a later date: stage 0, 0·2905; 15 days, 0·2955; hatched chick, 0·2716.

Exp. 2. Total N. Stage: 0 0·2912 g.
„ 15 days 0·2646
„ hatched chick 0·2464

	Amide	Humin	Diamino N Total	Amino	Non-amino	Argi-nine	Histi-dine	Ly-sine	Monoamino-N Total	Amino	Non-amino	Argi-nine
Stage: 0												
(1)	0·0280	0·0053	0·0700	0·0435	0·0265	0·0399	−0·0051	0·0352	0·1834	0·1730	0·0104	0·0112
(2)	0·0290	0·0055	0·0700	0·0371	0·0329	0·0371	0·0076	0·0253	0·1862	0·1730	0·0132	0·0112
Mean	0·0285	0·0054	0·0700	0·0403	0·0297	0·0385	0·0012	0·0303	0·1848	0·1730	0·0118	0·0112
Residual solution	—	—	0·0728	0·0366	0·0362	0·0385	0·0110	0·0233	0·1862	0·1728	0·0134	0·0126
Stage: 15 days												
(1)	0·0251	0·0052	0·0644	0·0293	0·0351	0·0343	0·0141	0·0160	0·1666	0·1512	0·0154	0·0098
(2)	0·0251	0·0052	0·0658	0·0288	0·0370	0·0336	0·0177	0·0145	0·0166	0·1502	0·0164	0·0084
Mean	0·0251	0·0052	0·0651	0·0290	0·0361	0·0340	0·0159	0·0152	0·0166	0·1507	0·0159	0·0091
Residual solution	—	—	0·0630	0·0307	0·0323	0·0343	0·0099	0·0188	0·0166	0·1517	0·0149	0·0084
Stage: hatched chick												
(1)	0·0216	0·0045	0·0658	0·0298	0·0360	0·0357	0·0138	0·0163	0·1568	0·1360	0·0208	0·0084
(2)	0·0214	0·0042	0·0630	0·0333	0·0297	0·0357	0·0044	0·0229	0·1540	0·1385	0·0155	0·0070
Mean	0·0215	0·0043	0·0644	0·0315	0·0329	0·0357	0·0092	0·0175	0·1554	0·1373	0·0181	0·0077
Residual solution	—	—	0·0658	0·0290	0·0368	0·0357	0·0150	0·0151	0·1540	0·1353	0·0187	0·0084

The sulphur estimations, in terms of cystine N, were:

	Cystine N			
	Exp. 1		Exp. 2	
	Diamino	Monoamino	Diamino	Monoamino
Stage: 0	0·0084	0·0161	0·0035	0·0114
„ 15 days	0·0082	0·0159	0·0031	0·0076
„ hatched chick	0·0126	0·0147	0·0014	0·0082

The amounts of bromine absorbed, and the corresponding amounts of tyrosine nitrogen, were:

	Exp. 1		Exp. 2	
	Br	Tyrosine N	Br	Tyrosine N
Stage: 0	0·516	0·0225	0·304	0·0132
„ 15 days	0·466	0·0203	0·278	0·0121
„ hatched chick	0·429	0·0187	0·224	0·0098

Expressed in terms of percentage of the total nitrogen the data come out as follows.

The two sets of figures giving a mean are from the mean result of the several analyses and from the result using the combined solutions which remained from the separate determinations.

	Exp. 1 Stage			Exp. 2 Stage		
	0	15 days	hatched	0	15 days	hatched
Amide	8·2	8·7	8·2	9·8	9·5	8·7
Humin	1·5	1·8	1·7	1·8	1·9	1·8
Diamino:						
Total	25·7, 26·0 } 25·8	26·7, 27·5 } 27·1	27·0, 27·8 } 27·4	24·0, 25·0 } 24·5	24·6, 23·8 } 24·2	26·1, 26·7 } 26·4
Amino	14·9, 14·7 } 14·8	15·5, 14·6 } 15·0	14·7, 14·2 } 14·5	13·8, 12·6 } 13·2	11·0, 11·6 } 11·3	12·8, 11·8 } 12·2
Non-amino	10·8, 11·3 } 11·0	11·1, 12·7 } 11·9	12·3, 13·6 } 13·0	10·2, 12·4 } 11·3	13·6, 12·2 } 12·9	13·3, 14·9 } 14·1
Arginine	13·9, 14·8 } 14·3	14·6, 15·3 } 15·0	16·6, 17·1 } 16·8	13·2, 13·2 } 13·2	13·5, 13·5 } 13·5	14·5, 14·5 } 14·5
Histidine	0·5, 0·3 } 0·4	0·3, 2·1 } 1·2	−0·1, 1·1 } 0·5	0·4, 3·8 } 2·1	6·0, 3·7 } 4·8	3·8, 6·1 } 4·9
Lysine	11·2, 10·9 } 11·0	11·7, 10·1 } 10·9	10·6, 9·5 } 10·2	10·4, 8·0 } 9·2	5·7, 7·1 } 6·4	7·1, 6·1 } 6·6
Cystine	1·8	1·7	2·6	1·2	1·2	0·6
Monoamino:						
Total	64·1, 63·9 } 64·0	61·0, 61·6 } 61·3	60·5, 60·0 } 60·2	63·4, 63·9 } 63·7	62·9, 62·9 } 62·9	63·1, 62·5 } 62·8
Amino	63·4	60·0	56·7	59·4, 59·3 } 59·3	56·9, 57·3 } 57·1	55·8, 54·9 } 55·3
Non-amino	0·6	1·3	4·5	4·1, 4·6 } 4·3	6·0, 5·6 } 5·8	7·3, 7·6 } 7·4
Arginine	4·6, 4·3 } 4·4	3·7, 3·4 } 3·6	3·3, 3·2 } 3·2	3·8, 4·3 } 4·0	2·9, 3·2 } 3·1	3·1, 3·4 } 3·3
Cystine	3·5	4·9	5·7	3·9	—	3·3

Discussion of results.

No change can be considered to occur in the amide and humin nitrogen, though a small decrease was observed in the amide nitrogen in the second experiment.

In both experiments a distinct increase occurred in the total diamino-nitrogen amounting to about 2 % of the total nitrogen of the egg proteins. Corresponding with this increase there was an increase in the amount of arginine nitrogen amounting to about 1 %. The figure in the first experiment with the hatched chick is probably high from error in the amide determination.

As has been previously experienced the estimation of the amino-nitrogen has given very erratic results; consequently no stress can be laid upon the figures for histidine and lysine; they probably increase, as the arginine increase only accounts for half the increase of the total diamino-nitrogen.

The total monoamino-nitrogen definitely decreased in both experiments. In the first experiment the amino-nitrogen figures were too high, but on repetition at a later date on the combined residues, they showed a decrease, as found in the second experiment. There was an increase in the non-amino-nitrogen.

Cystine nitrogen increased in both the diamino- and monoamino-fractions in the first experiment; in the second experiment the amounts of barium sulphate were so small that the figures are probably not correct.

The bromine absorption figures indicate a marked decrease during development. As the cystine nitrogen increases, this change probably indicates a decrease in the tyrosine content.

It would thus appear that in the metabolism of the developing egg the monoamino-acids are used for the formation of urea, uric acid, etc., and for furnishing energy, whilst the diamino-acids are retained for building the body protein of the chick. Loss of monoamino-acid would leave a remainder with a higher diamino-acid content. The changes run parallel with the changes which have been observed with salmon during the spawning season. These fish derive their energy from the monoamino-acids of the muscular tissue, whilst the diamino-acids pass to the reproductive organs. The eggs of the brook-trout and salamander have also been found [see Needham, 1925] to show an increase of diamino-nitrogen during development.

Summary.

Estimation of the amino-acids in the hen's egg during development by the modified Van Slyke method has shown that there is an increase of about 2 % in the diamino-nitrogen, of which the arginine increase makes about 1 % of the total nitrogen of the egg proteins. The monoamino-acid nitrogen decreases by about 4 % during development with a corresponding decrease in the amino-nitrogen content.

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REFERENCES.

Fiske and Boyden (1926). *J. Biol. Chem.* **70**, 535.
Needham (1925). *Physiol. Reviews*, 5. No. 1.
—— (1926, 1). *Brit. J. Exp. Biol.* **3**, 189.
—— (1926, 2). *Brit. J. Exp. Biol.* **4**, 114, 145.
Plimmer and Lowndes (1927). *Biochem. J.* **21**, 247.
Plimmer and Phillips (1924). *Biochem. J.* **18**, 312.
Plimmer and Rosedale (1925). *Biochem. J.* **19**, 1015.
Plimmer and Scott (1909). *J. Physiol.* **38**, 247.
Sendju (1925). *Japan. J. Biochem.* **5**, 391.

XXXVI. A NOTE ON THE ESTIMATION OF CHLORINE IN MILK.

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(*Received November 11th, 1926.*)

Austin and Van Slyke [1920] have shown that the Van Slyke-Donleavy [1919] method for the determination of plasma chlorides, when applied to whole blood, gives results too high by 30 to 40 %. If, however, the protein is precipitated by picric and nitric acids, and the protein-free filtrate is treated with silver nitrate, a quantitative precipitation of the total chloride of the whole blood is obtained and the final titration may be carried out as in the Van Slyke-Donleavy method.

Sisson and Dennis [1921], employing the Van Slyke-Donleavy method, with slight modifications, for the determination of chlorine in human milk, claim to have obtained results essentially similar to those obtained by fusion of the milk with sodium carbonate, to remove the organic material, and subsequent titration by the Volhard method. We have investigated the suitability of these two methods for the determination of chlorine in milk and have checked our results by means of Volhard titration after ashing the milk with sodium carbonate or with lime.

It was found, in testing these methods, that it was advisable to use the modified picric acid solution suggested by Sisson-Dennis to obtain complete precipitation of the milk proteins. The technique as used by us was as follows.

(*a*) *Method of Sisson-Dennis.* To 10 cc. of milk measured into a small flask were added 20 cc. of 1·2 % picric acid solution containing 2 cc. of glacial acetic acid per litre. The mixture was well shaken and allowed to stand for 5–10 minutes. 10 cc. of $N/10$ silver nitrate were then added, the mixture was again shaken and filtered on a chlorine-free filter paper. 20 cc. of clear filtrate were measured off and titrated against standard ammonium thiocyanate solution, as in the Volhard method.

(*b*) *Method of Austin and Van Slyke.* To 20 cc. of milk measured into a small flask were added 40 cc. of the picric acid solution, and after 10 minutes the curd was filtered off on a chlorine-free filter paper. 30 cc. of clear filtrate were then treated with 10 cc. of $N/10$ silver nitrate, the mixture was shaken, again filtered and 20 cc. of the clear filtrate were titrated against the standard thiocyanate.

The Volhard method for making the final titration was adopted for the sake of convenience.

The results obtained by us do not agree with the findings of Sisson-Dennis, but are in entire agreement with the results obtained by Austin and Van Slyke on whole blood.

In all the milks we tested the figures obtained were too high unless the protein was first removed before adding the silver nitrate solution. A comparison of the percentage of chlorine found by the different methods is shown in Table I.

Table I.

No. of sample	Animal	Method of Sisson-Dennis Cl %	Method of Austin-Van Slyke Cl %	Method of ashing Cl %
1	Cow	0·1276	0·1028	0·1028
2	,,	0·1276	0·1064	—
3	,,	0·1347	0·1064	—
4	Goat	0·1808	0·1560	—
5	,,	0·1454	0·1135	0·1135

The figures given in Table II indicate that when using the method of Sisson-Dennis the results are liable to vary if changes are made in the ratio of milk to silver nitrate used in the determination, whereas, by the method of Austin-Van Slyke, the percentage of chlorine found is independent of these changes provided that an excess of silver nitrate is present.

Table II. *Effect of varying the quantities of milk and silver nitrate.*

No. of sample	Amount of milk cc.	Amount of $AgNO_3$ cc. $N/10$	Method of Sisson-Dennis Cl %	Method of Austin-Van Slyke Cl %
1	10	10	0·1276	0·1064
1	20	10	0·1383	—
1	10	20	0·1312	—
2	10	10	0·1347	0·1064
2	5	10	—	0·1090
2	20	10	—	0·1064

The only effect of varying the amount of picric acid is to change the rate of filtration. In view of the fact that the presence of the protein of milk gives values too high when determining the chlorine content of milk a few tests were carried out on a chlorine-free solution of caseinogen using the methods described above.

Table III shows that only by the method of Sisson-Dennis was any apparent chlorine content found. This would appear to be due to either adsorption or combination of some of the silver by the precipitated protein.

Table III. *Test of methods on chlorine-free solution of caseinogen.*

Amount of caseinogen in solution g.	Apparent Cl-content found by method of Sisson-Dennis g.	Cl found by method of Van Slyke	Cl found by ashing with lime
0·3	0·00354	Nil	Nil
0·3	0·00213	,,	,,

Conclusion.

To determine accurately the chlorine content of milk volumetrically by precipitation of the chlorine as silver chloride, it is essential that the protein be removed before the addition of the silver nitrate.

REFERENCES.

Austin and Van Slyke (1920). *J. Biol. Chem.* **41**, 345.
Sisson and Dennis (1921). *Amer. J. Dis. Child.* **21**, 389.
Van Slyke and Donleavy (1919). *J. Biol. Chem.* **37**, 551.

XXXVII. THE BACTERIAL DECOMPOSITION OF TOBACCO AS LEADING TO THE FORMATION OF BASES IN THE PRESENCE OF WATER.

By A. FAITELOWITZ.

(*Received November 15th, 1926.*)

In work published in this *Journal* Fodor and Reifenberg [1925] came to the following conclusions.

(1) Tobacco extracts are capable of decomposing nicotine into pyridine and amino-bases, during which process a gradual increase in alkalinity takes place.

(2) The nicotine-decomposing activity of fermented tobacco is found to be smaller than that of non-fermented tobacco.

(3) Tobacco leaves dried artificially and quickly at 30° proved to be less active in decomposing nicotine than leaves dried slowly and naturally.

(4) The well-known amelioration of the quality of tobacco due to fermentation is attributed by Fodor and Reifenberg to the decomposition of a part of the nicotine through the action of an oxidase into pyridine and amino-bases. They consider that the aromatic qualities of tobacco are thus affected while denying the formation of acids during that process.

Numerous experiments are quoted with tobacco of different brands and origin which, however, appear to be hardly comparable among themselves, thus leaving the last three conclusions open to objection.

The first finding was confirmed by the writer, though this fact had to be differently interpreted in view of other discordant results.

For instance, the important rôle attributed by Fodor and Reifenberg to the decomposition of part of the nicotine into pyridine and amine with development of alkalinity is, in fact, quite insignificant for tobacco fermentation as demonstrated in the following preliminary qualitative tests. These tests rather indicate that the splitting up of nicotine and the subsequent increase in alkalinity gradually impair the quality of tobacco.

Experimental.

(*a*) To 10 g. of cigarette tobacco 90 cc. of water were added. After leaving the mixture to stand for 2 hours the macerate was separated from the tobacco by squeezing the latter in a cloth. All macerates so treated gave without exception an acid reaction to litmus paper. The acidity of macerates of

different brands varied from 15–20 cc. $N/10$ NaOH for 100 cc. of tobacco macerate.

(*b*) If a tobacco macerate is left to stand for a long time at room temperature (17–20°)—no matter whether filtered or not—then the acidity gradually begins to fall off and the macerate turns alkaline after about 30 hours' standing.

(*c*) On addition of 2 cc. chloroform to 10 cc. of a still acid tobacco macerate, kept in a well-stoppered bottle in order to prevent evaporation of chloroform, the reaction remains acid, while in another stoppered bottle the same macerate without addition of chloroform turns alkaline after a certain length of time.

From the foregoing experiments (*a*), (*b*) and (*c*) we learn that fermented cigarette tobacco (for cigarettes consist exclusively of fermented tobacco) does not become alkaline during the dry fermentation process, but does so if brought into contact with water. Such basic reaction should be attributed to bacterial action rather than to oxidase, for it is a well-established fact that chloroform does not interfere with enzymic reaction but arrests bacterial activity [Faitelowitz, 1910].

(*d*) Treatment of cigarette tobacco:

40 cc. of water are added to 10 g. of cigarette tobacco. The mixture is kept in a covered vessel at room temperature till the reaction turns basic. Then the pulp is spread out in a wide open dish and left to stand for some time (2–3 days) till the maccrate shrinks as the water evaporates. It is then left until a perfectly air dry condition is reached. Tobacco so treated becomes black, gives a basic reaction to litmus paper and evolves an odour probably due to pyridine and amino-bases. The tobacco is then unpleasant when smoked and has no value as cigarette tobacco.

As regards the dry curing process it will be the object of a further detailed study to determine the rate of development of acidity and catalase content in freshly cut tobacco leaves, in leaves dried slowly but not yet fermented, and in already fermented leaves. Should an increase of catalase and acidity be found in fermented leaves, then it would become apparent that tobacco fermentation is a similar process to milk fermentation where acid-forming bacteria produce lactic acid and catalase [see also Isajeff, 1904].

Summary.

1. The formation of pyridine and amino-bases from nicotine with a resulting basic reaction does not occur to any appreciable extent in the dry fermentation process.

2. In the presence of moisture the decomposition of tobacco leads to a basic reaction, but this condition reduces the quality of tobacco for the purpose of smoking.

3. The decomposition of the tobacco leaf in the presence of water is not due to enzymic action but most probably to the action of bacteria, since, in the presence of chloroform which eliminates bacterial action, the reaction of tobacco remains permanently acid.

REFERENCES.

Faitelowitz (1910). *Zentr. Milchwirt.* Nos. 7, 8 and 9.
Fodor and Reifenberg (1925). *Biochem. J.* **19**, 830.
Isajeff (1904). *Z. physiol. Chem.* **42**, 102.